Lela Buckingham ■ Maribeth L. Flaws

MOLECULAR DIAGNOSTICS

Fundamentals, Methods, & Clinical Applications

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Fundamentals, Methods, & Clinical Applications

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DEDICATION

To Zachary

LB

This textbook is especially dedicated to my parents Maureen and Edward Laude, my husband John, and our daughters Emily, Michelle, and Kristen. To my family, friends, teachers, students, and colleagues, I am forever grateful for your guidance, support, and encouragement.

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PREFACE

Molecular Technology has been implemented into diagnostic testing in a relatively short period. Programs that educate clinical laboratory professionals have had to incorporate molecular-based diagnostic testing into their curricula just as rapidly despite a lack of formal resources. This textbook was written to address these concerns.

The primary audience for this text is students enrolled in Clinical Laboratory Science/Medical Technology programs at all levels. The textbook explains the principles of molecular-based tests that are used for diagnostic purposes. Examples of applications of molecular-based assays are included in the text as well as case studies that illustrate the use and interpretation of these assays in patient care.

This textbook is also appropriate for students in other health-related disciplines who have to understand the purpose, principle, and interpretation of molecular-based diagnostic tests that they will be ordering and assessing for their patients.

Students who are first learning about molecular-based assays will find this text useful for explaining the principles. Practitioners who are performing and interpreting these assays can use this text as a resource for reference and trouble-shooting and to drive the implementation of additional molecular-based assays in their laboratory.

For educators who adopt this text for a course, we have developed an Instructor's Resource package. These

resources, which include a Brownstone test generator, image bank, and PowerPoint presentation, are available on CD-ROM and on Davis*Plus* at http://davisplus.fadavis.com. Educators should contact their F.A. Davis Sales Representative to obtain access to the Instructor's Resources.

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SECTION 1

Fundamentals of Nucleic Acid Biochemistry: An Overview

Chapter 1 *Lela Buckingham*

DNA

OUTLINE

DNA

DNA STRUCTURE
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RECOMBINATION IN ASEXUAL REPRODUCTION
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PLASMIDS

OBJECTIVES

- Diagram the structure of nitrogen bases, nucleosides, and nucleotides.
- Describe the nucleic acid structure as a polymer of nucleotides.
- Demonstrate how deoxyribonucleic acid (DNA) is replicated such that the order or sequence of nucleotides is maintained (semiconservative replication).
- Explain the reaction catalyzed by DNA polymerase that results in the phosphodiester backbone of the DNA chain.
- Note how the replicative process results in the antiparallel nature of complementary strands of DNA.
- List the enzymes that modify DNA, and state their specific functions.
- Illustrate three ways in which DNA can be transferred between bacterial cells.
- Define recombination, and sketch how new combinations of genes are generated in sexual and asexual reproduction.

When James Watson coined the term "molecular biology,"[1] he was referring to the biology of deoxyribonucleic acid (DNA). Of course, there are other molecules in nature. The term, however, is still used to describe the study of nucleic acids. In the clinical molecular laboratory, molecular techniques are designed for the handling and analysis of the nucleic acids, DNA and ribonucleic acid (RNA). Protein analysis and that of carbohydrates and other molecular species remain, for the most part, the domain of clinical chemistry. Molecular techniques are, however, being incorporated into other testing venues such as cell surface protein analysis, in situ histology, and tissue typing. The molecular biology laboratory, therefore, may be a separate entity or part of an existing molecular diagnostics or molecular pathology unit.

Nucleic acids offer several characteristics that support their use for clinical purposes. Highly specific analyses can be carried out through hybridization and amplification techniques without requirement for extensive physical or chemical selection of target molecules or organisms. This sensitivity allows specific and rapid analysis from limiting specimens. Furthermore, information carried in the sequence of the nucleotides that make up the DNA macromolecule is the basis for normal and pathological traits from microorganisms to humans and, as such, provides a valuable means of predictive analysis. Effective prevention and treatment of disease will result from the analysis of these sequences in the clinical laboratory.

Historical Highlights

Johann Friedrich Miescher is credited with the discovery of DNA in 1869.[2] Miescher had isolated white blood cells from seepage collected from discarded surgical bandages. He found that he could extract a viscous substance from these cells. Miescher also observed that most of the nonnuclear cell components could be lysed away with dilute hydrochloric acid, leaving the nuclei intact. Addition of extract of pig stomach (a source of pepsin to dissolve away contaminating proteins) resulted in a somewhat shrunken but clean preparation of nuclei. Extraction of these with alkali yielded the same substance isolated from the intact cells. It precipitated upon the addition of acid and redissolved in alkali. Chemical analysis of this substance demonstrated that it was 14% nitrogen and 2.5% phosphorus, different from any then known group of biochemicals. He named the substance "nuclein." (Analytical data indicate that less than 30% of Miescher's first nuclein preparation was actually DNA.) He later isolated a similar viscous material from salmon sperm and noted: "If one wants to assume that a single substance…is the specific cause of fertilization, then one should undoubtedly first of all think of nuclein."

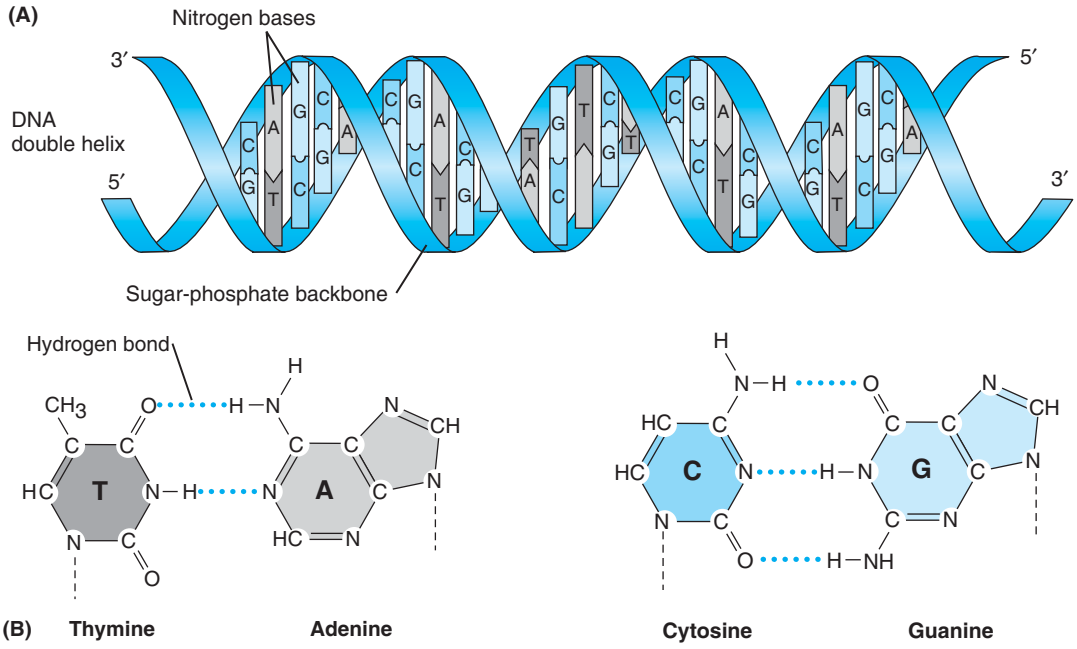
DNA

DNA is a macromolecule of carbon, nitrogen, oxygen, phosphorous, and hydrogen atoms. It is assembled in units or **nucleotides** that are composed of a phosphorylated ribose sugar and a **nitrogen base**. There are four nitrogen bases that make up the majority of DNA found in all organisms in nature. These are **adenine**, **cytosine**, **guanine**, and **thymine**. Nitrogen bases are attached to a **deoxyribose** sugar, which forms a polymer with the deoxyribose sugars of other nucleotides through a **phosphodiester bond**. Linear assembly of the nucleotides makes up one strand of DNA. Two strands of DNA comprise the DNA double helix.

In 1871, Miescher published a paper on **nuclein**, the viscous substance extracted from cell nuclei. In his writings, he made no mention of the function of nuclein. Walther Flemming, a leading cell biologist, describing his work on the nucleus in 1882 admitted that the biological significance of the substance was unknown. We now know that the purpose of DNA, contained in the nucleus of the cell, is to store information. The information in the DNA storage system is based on the order or sequence of nucleotides in the nucleic acid polymer. Just as computer information storage is based on sequences of 0 and 1, biological information is based on sequences of A, C, G, and T. These four building blocks (with a few modifications) account for all of the biological diversity that makes up life on Earth.

DNA Structure

The double helical structure of DNA (Fig. 1-1) was first described by James Watson and Francis Crick. Their



■ Figure 1-1 (A) The double helix. The phosphodiester backbones of the two nucleic acid chains form the helix. Nitrogen bases are oriented toward the center where they hydrogen-bond with homologous bases to stabilize the structure. (B) Two hydrogen bonds form between adenine and thymine. Three hydrogen bonds form between guanine and cytosine.

molecular model was founded on previous observations of the chemical nature of DNA and physical evidence including diffraction analyses performed by Rosalind Franklin.[3] The helical structure of DNA results from the physicochemical demands of the linear array of nucleotides. Both the specific **sequence** (order) of nucleotides in the strand as well as the surrounding chemical microenvironment can affect the nature of the DNA helix.

Nucleotides

The four nucleotide building blocks of DNA are molecules of about 700 kd. Each nucleotide consists of a five-carbon sugar, the first carbon of which is covalently joined to a nitrogen base and the fifth carbon to a triphosphate moiety (Fig. 1-2). A nitrogen base bound to an unphosphorylated sugar is a **nucleoside**. **Adenosine** (**A**), **guanosine** (**G**), **cytidine** (**C**), and **thymidine** (**T**) are nucleosides. If the ribose sugar is phosphorylated, the molecule is a nucleoside mono-, di-, or triphophosphate or a **nucleotide**. For example, adenosine with one phosphate is adenosine monophosphate (AMP). Adenosine with three phosphates is adenosine triphosphate (ATP). Nucleotides can be converted to nucleosides by hydrolysis. The five-carbon sugar of DNA is **deoxyribose**, which is ribose with the number two carbon of deoxyribose linked to a hydrogen atom rather than a hydroxyl group (see Fig. 1-2). The hydroxyl group on the third carbon is important for forming the phosphodiester bond that is the backbone of the DNA strand.

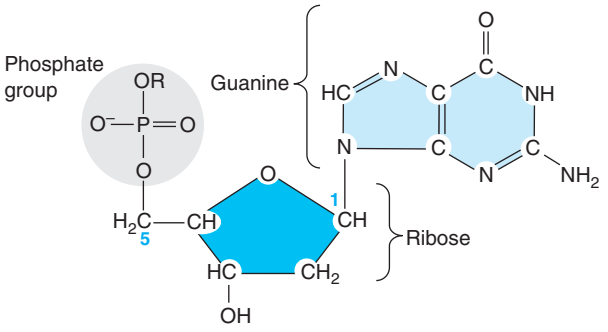
Nitrogen bases are planar carbon-nitrogen ring structures. The four common nitrogen bases in DNA are **adenine**, **guanine**, **cytosine**, and **thymine**. Amine and ketone substitutions around the ring as well as the single or double bonds within the ring distinguish the four bases that comprise the majority of DNA (Fig. 1-3). Nitrogen bases with a single ring (thymine, cytosine) are **pyrimidines**. Bases with a double ring (guanine, adenine) are **purines**.

Numbering of the positions in the nucleotide molecule starts with the ring positions of the nitrogen base, desig-

Advanced Concepts

The double helix first described by Watson and Crick is DNA in its hydrated form (B-form) and is the standard form of DNA.[4] It has 10.5 steps or pairs of nucleotides (bp) per turn. Dehydrated DNA takes the A-form with about 11 bp per turn and the center of symmetry along the outside of the helix rather than down the middle as it is in the B-form. Both A- and B-form DNA are right-handed helices. Stress and torsion can throw the double helix into a Z-form. Z-DNA is a left-handed helix with 12 bp per turn and altered geometry of the sugar-base bonds. Z-DNA has been observed in areas of chromosomes where the DNA is under torsional stress from unwinding for transcription or other metabolic functions.

Watson-Crick base pairing (purine:pyrimidine hydrogen bonding) is not limited to the ribofuranosyl nucleic acids, those found in our genetic system. Natural nucleic acid alternatives can also display the basic chemical properties of RNA and DNA. Theoretical studies have addressed such chemical alternatives to DNA and RNA components. An example is the pentopyranosyl-(2′→4′) oligonucleotide system that exhibits stronger and more selective base pairing than DNA or RNA.[5] Study of nucleic acid alternatives has practical applications. For example, protein nucleic acids, which have a carbon nitrogen peptide backbone replacing the sugar phosphate backbone,[6,7] can be used in the laboratory as alternatives to DNA and RNA hybridization probes.[8] They are also potential enzyme-resistant alternatives to RNA in antisense RNA therapies.[9]



dGMP

Figure 1-2 The nucleotide deoxyguanosine 5′ phosphate or guanosine monophosphate (dGMP). It is composed of deoxyribose covalently bound at its number 1 carbon to the nitrogen base, guanine, and at its number 5 carbon to a phosphate group. The molecule without the phosphate group is the nucleoside, deoxyguanosine.

nated C or N 1, 2, 3, etc. The carbons of the ribose sugar are numbered 1′ to 5′, distinguishing the sugar ring positions from those of the nitrogen base rings (Fig. 1-4).

The nitrogen base components of the nucleotides form **hydrogen bonds** with each other in a specific way. Guanine forms three hydrogen bonds with cytosine. Adenine forms two hydrogen bonds with thymine (see Fig. 1-1B). Hydrogen bonds between nucleotides are the key to the specificity of all nucleic acid–based tests used in the molecular laboratory. Specific hydrogen bond formation is also how the information held in the linear order of the nucleotides is maintained. As DNA is polymerized, each nucleotide to be added to the new DNA strand hydrogen bonds with the **complementary** nucleotide on the parental strand (A:T, G:C). In this way the parental DNA strand can be replicated without loss of the nucleotide order. Base pairs (bp) other than A:T and G:C or **mismatches**, e.g., A:C, G:T, can distort the DNA helix and disrupt the maintenance of sequence information.

Substituted Nucleotides

Modifications of the nucleotide structure are found throughout nature. Methylations, deaminations, additions, substitutions, and other chemical modifications generate nucleotides with new properties. Changes such as methylation of nitrogen bases have biological consequences for gene function and are intended in nature. Changes can

Advanced Concepts

In addition to the four commonly occurring nucleotide bases, **modified bases** are also often found in nature. Base modifications have significant affects on phenotype. Some modified bases result from damage to DNA; others are naturally modified for specific functions or to affect gene expression, as will be discussed in later sections.

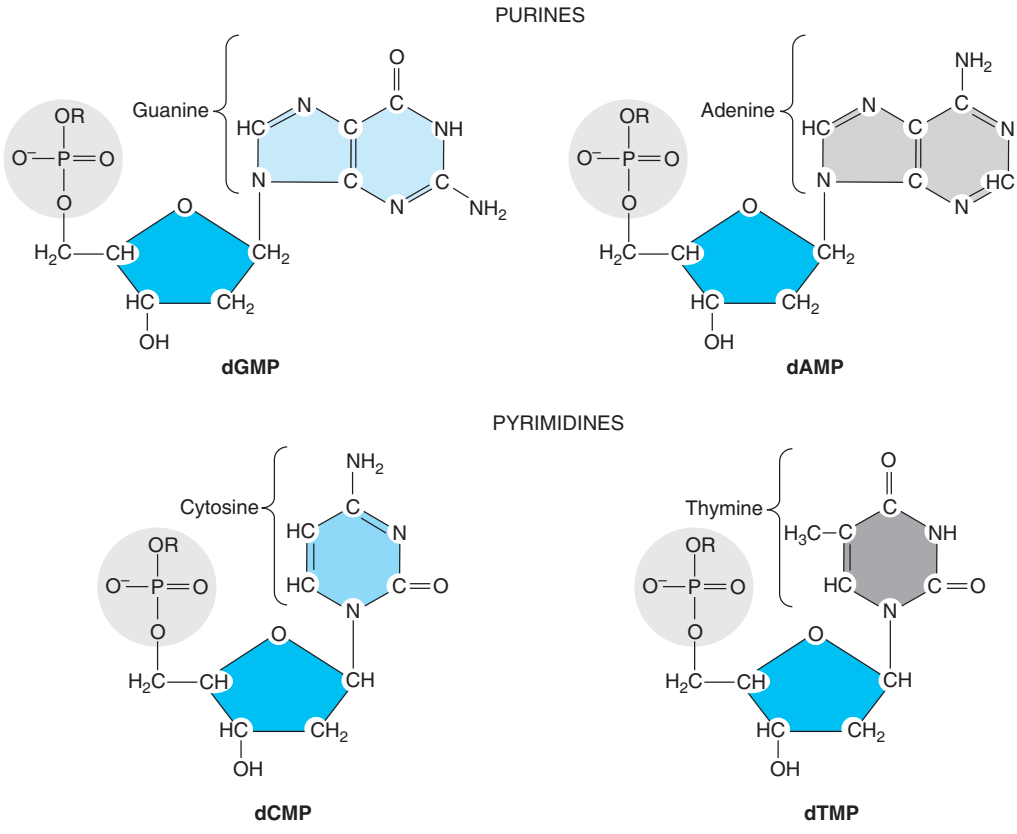


Figure 1-3 Nucleotides, deoxyguanosine monophosphate (dGMP), deoxyadenosine monophosphate (dAMP), deoxythymidine monophosphate (dTMP), and deoxycytidine monophosphate (dCMP), differ by the attached nitrogen bases. The nitrogen bases, guanine and adenine, have purine ring structures. Thymine and cytosine have pyrimidine ring structures. Uracil, the nucleotide base that replaces thymine in RNA, has the purine ring structure of thymine minus the methyl group and hydrogen bonds with adenine.

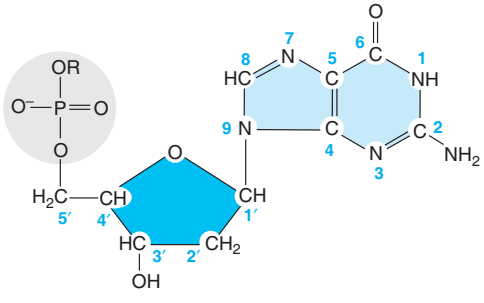


Figure 1-4 Carbon position numbering of a nucleotide monophosphate. The base carbons are numbered 1 through 9. The sugar carbons are numbered 1′ to 5′. The phosphate group on the 5′ carbon and the hydroxyl group on the 3′ carbon form phosphodiester bonds between bases.

also be brought about by environmental insults such as chemicals or radiation. These changes can affect gene function as well, resulting in undesirable effects such as cancer.

Advanced Concepts

Modified nucleotides are used in bacteria and viruses as a primitive immune system that allows them to distinguish their own DNA from that of host or invaders (**restriction modification [rm]** system). Recognizing its own modifications, the host can target unmodified DNA for degradation.

Due to their specific effects on enzymes that metabolize DNA, modified nucleosides have been used effectively for clinical applications (Fig. 1-5). The anticancer drugs, 5-bromouridine (5BrdU) and cytosine arabinoside (cytarabine, ara-C), are modified thymidine and cytosine nucleosides, respectively. Azidothymidine (Retrovir, AZT), cytosine, 2′,3′-dideoxy-2′-fluoro (ddC), and 2′,3′-dideoxyinosine (Videx, ddI), drugs used to treat patients with human immunodeficiency virus (HIV) infections, are modifications of thymidine and cytosine and a precursor of adenine, respectively. An analog of guanosine, 2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6*H*-purin-6-one (Acyclovir, Zovirax), is a drug used to combat herpes simplex virus and varicella-zoster virus.

In the laboratory, nucleosides can be modified for purposes of labeling or detection of DNA molecules, sequencing, and other applications. The techniques used for these procedures will be discussed in later chapters.

Nucleic Acid

Nucleic acid is a macromolecule made of nucleotides bound together by the phosphate and hydroxyl groups on their sugars. A nucleic acid chain grows by the attachment of the 5′ phosphate group of an incoming nucleotide to the 3′ hydroxyl group of the last nucleotide on the growing chain (Fig. 1-6). Addition of nucleotides in this way gives the DNA chain a **polarity**; that is, it has a 5′ phosphate end and a 3′ hydroxyl end. We refer to DNA as oriented in a **5′ to 3′ direction**, and the linear sequence of the nucleotides, by convention, is read in that order.

DNA found in nature is mostly **double-stranded**. Two strands exist in opposite 5′ to 3′/3′ to 5′ orientation held together by the hydrogen bonds between their respective bases (A with T and G with C). The bases are positioned such that the sugar-phosphate chain that connects them (**sugar-phosphate backbone**) is oriented in a spiral or helix around the nitrogen bases (see Fig. 1-1).

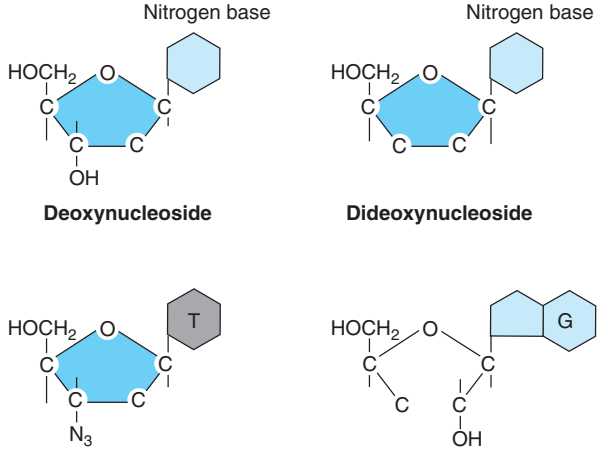


Figure 1-5 Substituted nucleosides used in the clinic and the laboratory.

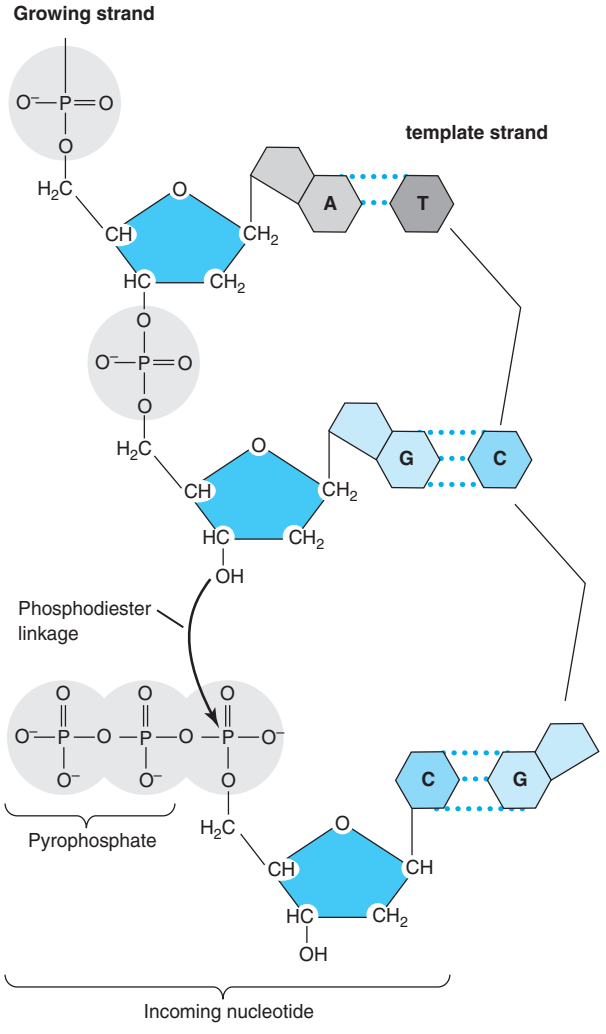


Figure 1-6 DNA replication is a template guided polymerization catalyzed by DNA polymerase.

Advanced Concepts

The sugar-phosphate backbones of the helix are arranged in specific distances from one another in the double helix (see Fig. 1-1). The two regions of the helix formed by the backbones are called the **major groove** and **minor groove**. The major and minor grooves are sites of interaction with the many proteins that bind to specific nucleotide sequences in DNA (**binding** or **recognition sites**). The double helix can also be penetrated by **intercalating agents**, molecules that slide transversely into the center of the helix. **Denaturing agents** such as formamide and urea displace the hydrogen bonds and separate the two strands of the helix.

The DNA double helix represents two versions of the information stored in the form of the order or **sequence** of the nucleotides on each chain. The sequences of the two strands that form the double helix are **complementary**, not identical (Fig. 1-7). They are in **antiparallel** orientation with the 5′ end of one strand at the 3′ end of the other (Fig. 1-8). Identical sequences will not hybridize with each other. In later sections we will appreciate the importance of this when designing hybridization and amplification assays.

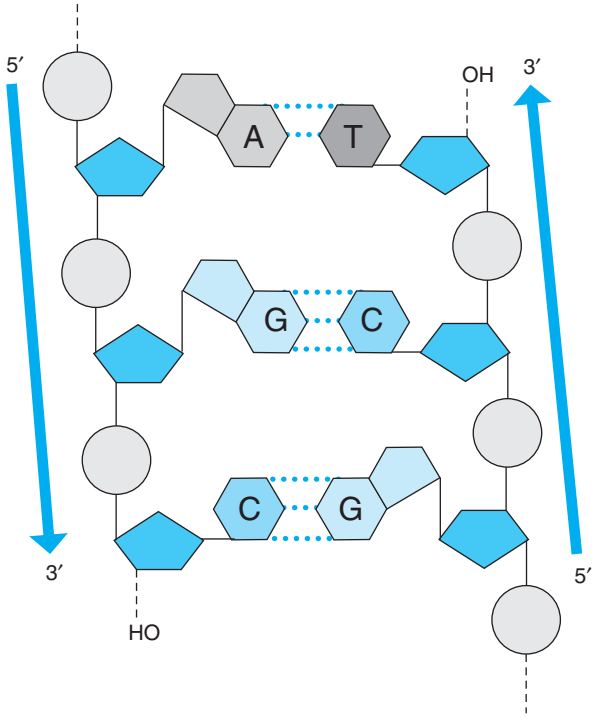


Figure 1-8 DNA synthesis proceeds from the 5′ phosphate group to the 3′ hydroxyl group. The template strand is copied in the opposite (3′ to 5′) direction. The new double helix consists of the template strand and the new daughter strand oriented in opposite directions from one another.

DNA Replication

DNA has an antiparallel orientation because of the way it is synthesized. As DNA synthesis proceeds in the 5′ to 3′ direction, DNA polymerase, the enzyme responsible for polymerizing the nucleotides, uses a guide, or **template**, to determine which nucleotides to add. The enzyme reads the template in the 3′ to 5′ direction. The resulting double strand, then, will have a parent strand in one orientation and a newly synthesized strand oriented in the opposite orientation.

PO 5′ **G T A G C T C G C T G A T** 3′ OH
HO 3′ **C A T C G A G C G A C T A** 5′ OP

Figure 1-7 Homologous sequences are not identical and are oriented in opposite directions.

As Watson and Crick predicted, **semiconservative** replication is the key to maintaining the sequence of the nucleotides in DNA through new generations. Every cell in a multicellular organism or in a clonal population of unicellular organisms carries the same genetic information. It is important that this information, in the form of the DNA sequence, be transferred faithfully at every cell division. The replication apparatus is designed to copy the DNA strands in an orderly way with minimal errors before each cell division.

The order of nucleotides is maintained because each strand of the parent double helix is the template for a newly replicated strand. In the process of replication, DNA is first unwound from the duplex so that each single strand may serve as a template for the order of addition of nucleotides to the new strand (see Fig. 1-6). The new strand is elongated by hydrogen bonding of the

Historical Highlights

Before the double helix was determined, Erwin Chargaff[10] made the observation that the amount of adenine in DNA corresponded to the amount of thymine and the amount of cytosine to the amount of guanine. Upon the description of the double helix, Watson proposed that the steps in the ladder of the double helix were pairs of bases, thymine with adenine and guanine with cytosine. Watson and Crick, upon publication of their work, suggested that this arrangement was the basis for a copying mechanism. The complementary strands could separate and serve as guides of templates for producing like strands.

proper incoming nucleotide to the nitrogen base on the template strand and then a nucleophilic attack of the deoxyribose 3′ hydroxyl oxygen on a phosphorous atom of the phosphate group on the hydrogen-bonded nucleotide triphosphate. Orthophosphate is released with the formation of a **phosphodiester bond** between the new nucleotide and the last nucleotide of the growing chain. The duplicated helix will ultimately consist of one template strand and one new strand.

DNA replication proceeds through the DNA duplex with both strands of DNA replicating in a single pass. DNA undergoing active replication can be observed by electron microscopy as a forked structure, or **replication fork**. Note, however, that the antiparallel nature of duplex DNA and the requirement for the DNA synthesis apparatus to read the template strand in a 3′ to 5′ direction are not consistent with copying of both strands simultaneously in the same direction. The question arises as to how one of the strands of the duplex can be copied in the same direction as its complementary strand that runs antiparallel to it. This problem was addressed in 1968 by Okazaki and Okazaki[12] studying DNA replication in *Escherichia coli*. In their experiments, small pieces of DNA, about 1000 bases in length, could be observed by density gradient centrifugation in actively replicating DNA. The fragments chased into larger pieces with time, showing that they were covalently linked together shortly after synthesis. These small fragments, or **Okazaki fragments**, were the key to explaining how both strands were copied at the replication fork. The two strands of the parent helix are

Historical Highlights

A few years after solution of the double helix, the mechanism of semiconservative replication was demonstrated by Matthew Meselson and Franklin Stahl,[11] using the technique of equilibrium density centrifugation on a cesium gradient. They prepared "heavy" DNA by growing bacteria in a medium containing the nitrogen isotope ^{15}N. After shifting the bacteria into a medium of normal nitrogen (^{14}N), they could separate the hybrid ^{14}N:^{15}N DNA molecules synthesized as the bacteria replicated. These molecules were of intermediate density to the ones from bacteria grown only in ^{14}N or ^{15}N. They could differentiate true semiconservative replication from **dispersive** replication by demonstrating that approximately half of the DNA double helices from the next generation grown in normal nitrogen were ^{14}N:^{15}N and half were ^{14}N:^{14}N.

not copied in the same way. The replication apparatus jumps ahead a short distance (~1000 bases) on the 5′ to 3′ strand and then copies backward toward the replication fork, while DNA replication proceeds in a continuous manner on the 3′ to 5′ strand, or the **leading strand**. The 5′ to 3′ strand copied in a discontinuous manner is the **lagging strand**[13] (Fig. 1-9).

Another requirement for DNA synthesis is the availability of the deoxyribose 3′ hydroxyl oxygen for chain growth. This means that DNA cannot be synthesized de novo. A preceding base must be present to provide the hydroxyl group. This base is provided by another enzyme component of the replication apparatus, **primase**. Primase is a ribonucleic acid (RNA) synthesizing enzyme that lays down short (6–11 bp) RNA primers required for priming DNA synthesis. Primase must work repeatedly on the lagging strand to prime synthesis of each Okazaki fragment.

Polymerases

The first purified enzyme shown to catalyze DNA replication in prokaryotes was designated **DNA polymerase I** (**pol I**). DNA polymerases II (pol II) and III were later characterized, and it was discovered that **DNA poly-**

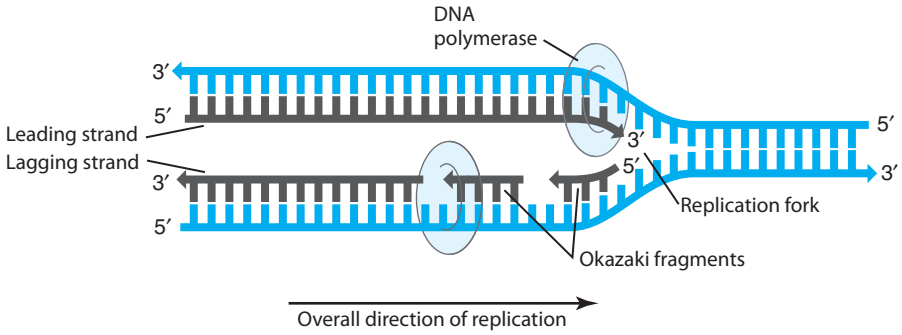


Figure 1-9 Simultaneous replication of both strands of the double helix. Both strands are read in the 3′ to 5′ direction. The lagging strand is read discontinuously, with the polymerase skipping ahead and reading back toward the replication fork on the lagging strand.

merase III (**pol III**) was the main polymerizing enzyme during bacterial replication (Table 1.1). The other two polymerases were responsible for repair of gaps and discontinuities in previously synthesized DNA. It not surprising that pol I was preferentially purified in those early studies. In in vitro studies where the enzymes were first described, pol II and pol III activity was less than 5% of that of pol I. In vivo, pol III functions as a multisubunit **holoenzyme**. The holoenzyme works along with a larger assembly of proteins required for priming, initiation, regulation, and termination of the replication process (Fig. 1-10). Two of the 10 subunits of the holoenzyme are catalytic DNA polymerizing enzymes, one for leading and one for lagging strand synthesis.[14]

Most DNA polymerase functions include, in addition to polymerization, **pyrophosphorolysis** and **pyrophos-**

Advanced Concepts

The DNA replication complex (**replisome**) contains all the necessary proteins for the several activities involved in faithful replication of double-stranded DNA. **Helicase** activity in the replisome unwinds and untangles the DNA for replication. Primase, either as a separate protein or in a **primase-helicase polyprotein** in the replisome, synthesizes short (11 ± 1 bases) RNA sequences to prime DNA synthesis. Primase activity is required throughout the replication process to prime the discontinuous synthesis on the lagging DNA strand. The *E. coli* primase, DnaG, transcribes 2000–3000 RNA primers at a rate of 1 per second in the replication of the *E. coli* genome. Separate polymerase proteins add incoming nucleotides to the growing DNA strands of the replication fork. The details of synthesis of the lagging strand are not yet clear, although recent evidence suggests discontinuous replication proceeds by a ratcheting mechanism, with replisome molecules pulling the lagging strand in for priming and copying. Once DNA is primed and synthesized, **Rnase H**, an enzyme that hydrolyzes RNA from a complementary DNA strand, removes the primer RNA from the short RNA-DNA hybrid, and the resulting gap is filled by another DNA polymerase, pol I.

Historical Highlights

At a conference on the chemical basis of heredity held at Johns Hopkins University in June 1956, Arthur Kornberg, I. Robert Lehman, and Maurice J. Bessman reported on an extract of *E. coli* that could polymerize nucleotides into DNA.[13] It was noted that the reaction required preformed DNA and all four nucleotides along with the bacterial protein extract. Any source of preformed DNA would work, bacterial, viral, or animal. At the time it was difficult to determine whether the new DNA was a copy of the input molecule or an extension of it. During the next 3 years, Julius Adler, Sylvy Kornberg, and Steven B. Zimmerman showed that the new DNA had the same A-T to G-C base pair ratio as the input DNA, and was indeed a copy of it. This ratio was not affected by the proportion of free nucleotides added to the initial reaction, confirming that the input or template DNA determined the sequence of the nucleotides on the newly synthesized DNA.

Table 1.1 Examples of Polymerases Classified by Sequence Homology

Family	Polymerase	Source	Activity
A	Pol I	*E. coli*	Recombination, repair, replication
A	T5 pol, T7 pol	T5, T7 bacteriophage	Replication
A	Pol γ	Mitochondria	Replication
B	Pol II	*E. coli*	Repair
B	Archael	*P. furiosus*	Replication, repair
B	ϕ29 pol, T4 pol	ϕ29, T4 bacteriophage	Replication
B	Polα, PolΔ, Polϵ	Eukaryotes	Repair
B	Viral pols	Various viruses	Repair
C	Pol III core	*E. coli*	Replication
C	dnaE, $dnaE_{BS}$	*B. subtilis*	Replication
X	Pol β	Eukaryotes	Repair, replication
?	Pol η, Pol τ	Eukaryotes	Bypass replication
?	Pol κ	Eukaryotes	Bypass replication, cohesion
?	Pol IV, Pol V	*E. coli*	Bypass replication
?	Rev1, Rad30	*S. cerevisiae*	Bypass replication
?	Rad 6, Pol ξ	*S. cerevisiae*	uv-induced repair

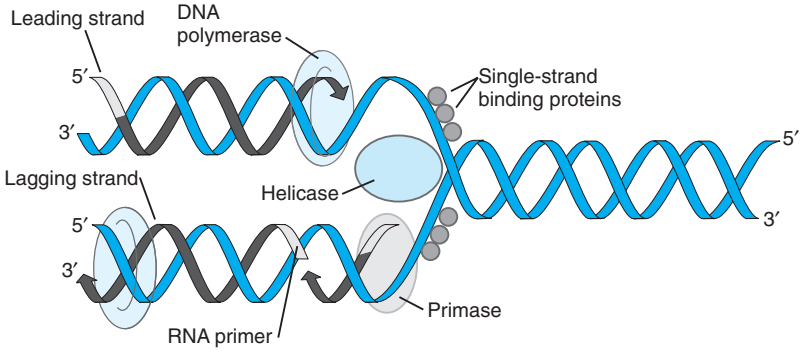


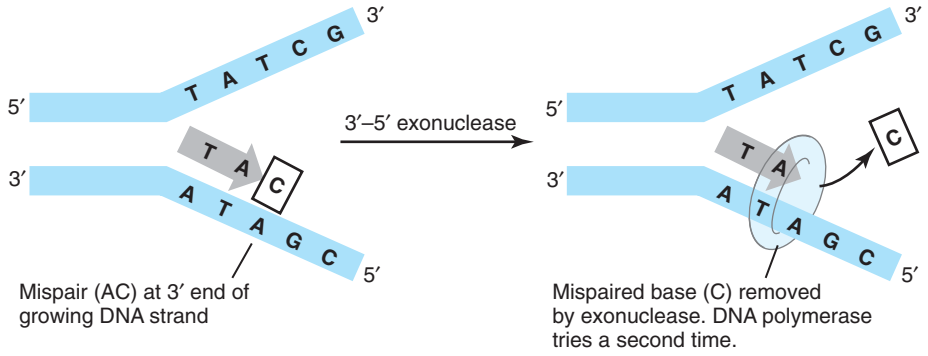
Figure 1-10 DNA polymerase activity involves more than one protein molecule. Several cofactors and accessory proteins are required to unwind the template helix (green), prime synthesis with RNA primers (gray), and protect the lagging strand (dark gray).

Advanced Concepts

Genome sequencing has revealed that the organization of the proteins in and associated with the holoenzyme is similar in bacteria of the *Bacillus/Clostridium* group and in the unrelated thermophile, *Thermotoga maritima.*[14] The conserved nature of the polymerase complex suggests a limited range of possible structures with polymerase activity. It also explains how a bacterial polymerase can replicate DNA from diverse sources. This is important in the laboratory where prokaryote polymerases are used extensively to copy DNA from many different sources.

phate exchange, the latter two activities being a reversal of the polymerization process. DNA polymerase enzymes thus have the capacity to synthesize DNA in a 5′ to 3′ direction and degrade DNA in both a 5′ to 3′ and 3′ to 5′ direction (Fig 1-11). The catalytic domain of *E. coli* DNA pol I can be broken into two fragments, separating the two functions, a large fragment carrying the polymerase activity and a small fragment carrying the exonuclease activity. The large fragment without the exonuclease activity (**Klenow fragment**) has been used extensively in the laboratory for in vitro DNA synthesis.

One purpose of the **exonuclease** function in the various DNA polymerases is to protect the sequence of nucleotides, which must be faithfully copied. Copying errors will result in base changes or **mutations** in the



■ Figure 1-11 DNA polymerase can remove misincorporated bases during replication using its 3′ to 5′ exonuclease activity.

DNA. The 3′ to 5′ exonuclease function is required to assure that replication begins or continues with a correctly base-paired nucleotide. The enzyme will remove a mismatch (for example, A opposite C instead of T on the template) in the primer sequence before beginning polymerization. During DNA synthesis, this exonuclease function gives the enzyme the capacity to **proofread** newly synthesized DNA; that is, to remove a misincorporated nucleotide by breaking the phosphodiester bond and replace it with the correct one.

During DNA replication, *E. coli* DNA pol III can synthesize and degrade DNA simultaneously. At a **nick**, or discontinuity, in one strand of a DNA duplex, the enzyme can add nucleotides at the 3′ end of the nick while removing nucleotides ahead of it with its 5′ to 3′ exonuclease function (Fig. 1-12). This concurrent synthesis and hydrolysis then move the nick in one strand of the DNA forward in an activity called **nick translation**. The polymerization and hydrolysis will proceed for a short distance until the polymerase is dislodged. The nick can then be reclosed by DNA **ligase**, an enzyme that forms phosphodiester bonds between existing DNA strands. Nick translation is often used in vitro as a method to introduce labeled nucleotides into DNA molecules. The resulting labeled products are used for DNA detection in hybridization analyses.

Another type of DNA polymerase, **terminal transferase**, can synthesize polynucleotide chains de novo without a template. This enzyme will add nucleotides to the end of a DNA strand in the absence of hydrogen base pairing with a template. The initial synthesis of a large dA-dT polymer by terminal transferase was a significant event in the history of DNA polymerase studies.[15] Terminal transferase is used in the laboratory to generate 3′-labeled DNA species.

Advanced Concepts

Like prokaryotes, eukaryotic cells contain multiple polymerase activities. Two polymerase protein complexes, designated α and β, are found in the nucleus and one, γ, in the mitochondria. The three polymerases resemble prokaryotic enzymes, except they have less demonstrable exonuclease activity. A fourth polymerase, δ, originally isolated from bone marrow, has 3′ to 5′ exonuclease activity. Polymerase α, the most active, is identified with chromosome replication, and β and δ are associated with DNA repair.

Advanced Concepts

After replication, distortions in the DNA duplex caused by mismatched or aberrantly modified bases are removed by the 5′ to 3′ exonuclease function of **repair** polymerases such as DNA pol I. This activity degrades duplex DNA from the 5′ end and can also cleave diester bonds several bases from the end of the chain. It is important for removing lesions in the DNA duplex such as **thymine** or **pyrimidine dimers**, boxy structures formed between adjacent thymines or cytosines and thymines on the same DNA strand that are induced by exposure of DNA to ultraviolet light. If these structures are not removed, they can disrupt subsequent transcription and replication of the DNA strand.

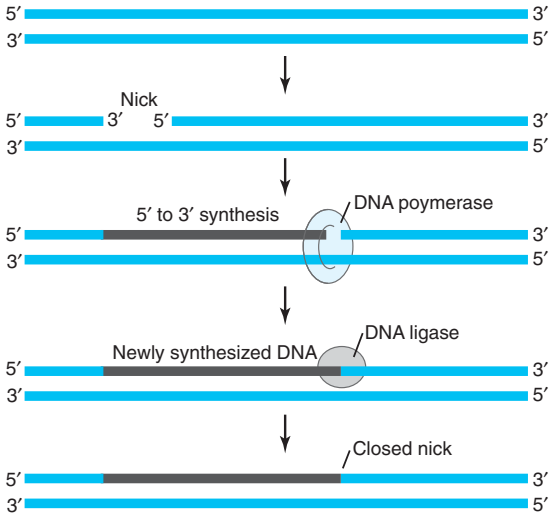


Figure 1-12 Nick translation of DNA. DNA polymerase extends the 3′ end of a nick in double-stranded DNA with newly synthesized strand (gray) while digesting the original strand from the 5′ end. After polymerization, the nick is closed by DNA ligase.

DNA polymerases play a central role in modern biotechnology. Cloning as well as some amplification and sequencing technologies all require DNA polymerase activity. The prerequisite for specific polymerase characteristics has stimulated the search for new polymerases and the engineering of available polymerase enzymes. Polymerases from various sources were classified into families (A, B, C, X) based on sequence structure.[16,17] Short summary is shown in Table 1.1. Other classifications are based on similarities in protein structure.

Polymerases in the A and B family are most useful for biotechnological engineering, as the polymerase activity of these enzymes is contained in a **monomeric** or single protein. Chemical manipulation of the amino acid structure of these enzymes produces polymerases with characteristics that are useful in the laboratory. These include altered **processivity** (staying with the template longer to make longer products), **fidelity** (faithful copying of the template), and **substrate specificity** (affinity for altered nucleotides).[19]

Enzymes That Metabolize DNA

Once DNA is polymerized, it is not static. The information stored in the DNA must be tapped selectively to make RNA and, at the same time, protected from mutation. In addition, an important aspect of reproduction is mixing of sequence information to generate genetic diversity (hybrid vigor) in the offspring, which requires cutting and reassembly of the DNA strands in advance of cell division and gamete formation. A host of enzymes performs these and other functions during various stages of the cell cycle. Some of these enzymes, including DNA polymerase, have been isolated for in vitro manipulation of DNA in the laboratory. They are key tools of recombinant DNA technology, the basis for commonly used molecular techniques.

Restriction Enzymes

Genetic engineering was stimulated by the discovery of deoxyriboendonucleases, or **endonucleases**. Endonucleases break the sugar phosphate backbone of DNA at internal sites.

Restriction enzymes are endonucleases that recognize specific base sequences and break or restrict the DNA

Advanced Concepts

Polymerases replicate DNA under different cellular conditions as shown in Table 1.1. A large part of DNA synthesis activity in the cell occurs after replication of the cellular DNA is complete. New information as to the nature of these enzymes indicates that polymerases can participate in cohesion (holding together) of sister chromatids to assure proper recombination and segregation of chromosomes.[18]

Advanced Concepts

These enzymes are of several types. Some prefer single-stranded and some prefer double-stranded DNA. **Repair endonucleases** function at areas of distortion in the DNA duplex such as baseless (apurinic or apyrimidic) sites on the DNA backbone, thymine dimers, or mismatched bases. As the chemical structure of DNA is the same in all organisms, most enzymes are active on DNA from diverse sources.

polymer at the sugar-phosphate backbone. These enzymes were originally isolated from bacteria where they function as part of a primitive defense system to cleave foreign DNA entering the bacterial cell. The ability of the cell to recognize foreign DNA depended on both DNA sequence recognition and methylation.

Restriction enzymes are named for the organism from which they were isolated. For example, *Bam*HI was isolated from *Bacillus amyloliquefaciens* H, *Hind*III from *Haemophilus influenzae* Rd, *Sma*I from *Serratia marcescens* Sb_b and so forth.

Restriction endonucleases have been classified into three types. **Type I restriction enzymes** have both nuclease and methylase activity in a single enzyme. They bind to host-specific DNA sites of 4–6 bp separated by 6–8 bp and containing methylated adenines. The site of cleavage of the DNA substrate can be over 1000 bp from this binding site. An example of a type I enzyme is *EcoK* from *E. coli* K 12. It recognizes the site:

5′ - A C N N N N N N G T G C
T G N N N N N N C A C G - 5′

where N represents nonspecific nucleotides and the adenine residues (A) are methylated.

Type III restriction enzymes resemble type I enzymes in their ability to both methylate and restrict (cut) DNA. Like type I, they are complex enzymes with two subunits. Recognition sites for these enzymes are asymmetrical, and the cleavage of the substrate DNA occurs 24–26 bp from the site to the 3′ side. An example of a type III enzyme is *Hinf*III from *H. influenzae*. It recognizes the site:

5′ - C G A A T
G C T T A - 5′

where the adenine methylation occurs on only one strand.

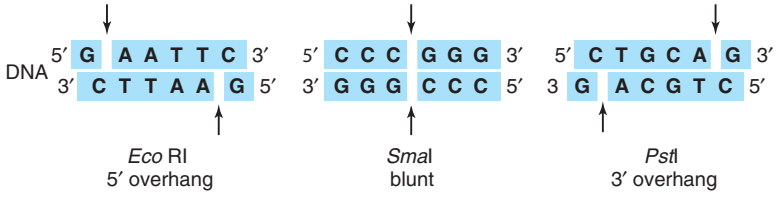
Type II restriction enzymes are those used most frequently in the laboratory. These enzymes do not have inherent methylation activity in the same molecule as the nuclease activity. They bind as simple dimers to their symmetrical DNA recognition sites. These sites are **palindromic** in nature; that is, they read the same 5′ to 3′ on both strands of the DNA (Fig. 1-13), referred to as **bilateral symmetry**. Type II restriction enzymes cleave the DNA directly at their binding site, producing fragments of predictable size.

Advanced Concepts

Restriction enzyme recognition sequences in the DNA are generally areas of bilateral rotational symmetry around an axis perpendicular to the DNA helix. The enzymes bind to the recognition site, which is usually 4–8 bp in length, as dimers to form a complex with twofold symmetry. The enzymes then cleave the DNA backbone at sites symmetrically located around the same twofold axis.

Type II restriction enzymes have been found in almost all prokaryotes, but none, to date, have been found in eukaryotes. The specificity of their action and the hundreds of enzymes available that recognize numerous sites are key factors in the ability to perform DNA recombination in vitro. Cutting DNA at specific sequences is the basis of many procedures in molecular technology, including mapping, cloning, genetic engineering, and mutation analysis. Restriction enzymes are frequently used in the clinical laboratory, for example, in the analysis of gene rearrangements and in mutation detection.

Although all type II restriction enzymes work with bilateral symmetry, their patterns of double-stranded breaks differ (see Fig. 1-13). Some enzymes cut the duplex with a **staggered** separation at the recognition site, leaving 2–4 base single-strand **overhangs** at the ends of the DNA. The single-strand ends can hybridize with complementary ends on other DNA fragments, directing the efficient joining of cut ends. Because of their ability to form hydrogen bonds with complementary overhangs, these cuts are said to produce "**sticky ends**" at the cut site. Another mode of cutting separates the



■ Figure 1-13 Restriction enzymes recognize symmetrical DNA sequences and cut the sugar phosphate background in different ways. Exposed single-stranded ends are "sticky" ends that can hybridize with complementary overhangs.

Advanced Concepts

The advantage of blunt ends for in vitro recombination is that blunt ends formed by different enzymes can be joined, regardless of the recognition site. This is not true for sticky ends, which must have matching overhangs. Sticky ends can be converted to blunt ends using DNA polymerase to extend the recessed strand in a sticky end, using the nucleotides of the overhang as a template or by using a single strand exonuclease to remove the overhanging nucleotides. Synthetic short DNA fragments with one blunt end and one sticky end (**adaptors**) can be used to convert blunt ends to specific sticky ends.

DNA duplex at the same place on both strands, leaving flush, or **blunt**, ends. These ends can be rejoined as well, although not as efficiently as sticky ends.

Restriction enzymes can be used for mapping a DNA fragment, as will be described in later sections. The collection of fragments generated by digestion of a given DNA fragment, e.g., a region of a human chromosome, with several restriction enzymes will be unique to that DNA. This is the basis for forensic identification and paternity testing using restriction fragment analysis of human DNA.

DNA Ligase

DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 3′-hydroxyl and 5′-phosphoryl nucleotide ends. Its existence was predicted by the observation of replication, recombination, and repair activities in vivo. These operations require reunion of the DNA backbone after discontinuous replication on the lagging strand, strand exchange, or repair synthesis. In 1967, DNA ligase was discovered in five different laboratories.[20] The isolated enzyme could catalyze end to end cleavage of separated strands of DNA.

Other DNA Metabolizing Enzymes

Other Nucleases

In contrast to endonucleases, **exonucleases** degrade DNA from free 3′ hydroxyl or 5′ phosphate ends. Consequently, they will not work on closed circular DNA. These enzymes are used, under controlled conditions, to manipulate DNA in vitro,[23] for instance to make stepwise deletions in linearized DNA or to modify DNA ends after cutting with restriction enzymes. Exonucleases have different substrate requirements and will therefore degrade specific types of DNA ends.

Exonuclease I from *E. coli* degrades single-stranded DNA from the 3′ hydroxyl end, producing mononucleotides. Its activity is optimal on long single-stranded ends, slowing significantly as it approaches a double-stranded region.

Exonuclease III from *E. coli* removes 5′ mononucleotides from the 3′ end of double-stranded DNA in the presence of Mg^{2+} and Mn^{2+}. It also has some endonuclease activity, cutting DNA at apurinic sites. Exo III removes nucleotides from blunt ends, recessed ends, and nicks, but will not digest 3′ overhangs. Exo III has been used in the research setting to create nested deletions in double-stranded DNA or to produce single-stranded DNA for dideoxy sequencing.

Exonuclease VII from *E. coli* digests single-stranded DNA from either the 5′ phosphate or 3′ hydroxyl end. It is one of the few enzymes with 5′ exonuclease activity. Exo VII can be employed to remove long single strands protruding from double-stranded DNA.

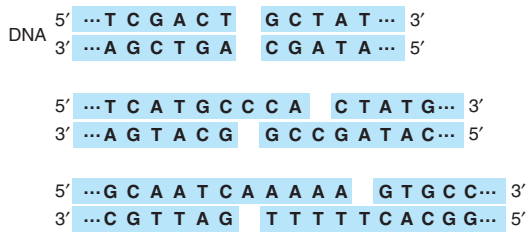
Nuclease Bal31 from *Alteromonas espejiani* can degrade single- and double-stranded DNA from both ends. Because its activity at 20°C is slow enough to control with good resolution, it has been used extensively in research applications to make nested deletions in DNA.

Advanced Concepts

DNA ligase can join both DNA and RNA ends. **RNA ligase**, first found in phage T4–infected bacteria, has the same activity. DNA ligases are more efficient in joining DNA ends and have been found in a wide variety of bacteria. The ability to convert open or nicked circles of DNA to closed circles, to protect free DNA ends, to extend DNA into an overhanging template, and to recover transformation-capable DNA after nicking are all activities of DNA ligase that led to its discovery and isolation.

Historical Highlights

The initial analysis of the joining reaction was performed with physically fractured DNA helices that had no homology at their ends. The joining reaction required the chance positioning of two adjacent ends and was, therefore, not very efficient. A better substrate for the enzyme would be ends that could be held together before ligation, i.e., by hydrogen bonds between single strands. H. Gobind Khorana showed that short synthetic segments of DNA with single-strand complementary overhangs joined into larger fragments efficiently.[21]Several investigators observed the increased efficiency of joining of ends of DNA molecules from certain bacterial viruses. These ends have naturally occurring single-stranded overhangs. It was also observed that treatment of DNA ends with **terminal transferase** to add short runs of A's to one fragment and T's to another increased the efficiency of joining ends of any two treated fragments. Although not yet available when ligase activity was being studied, it was subsequently observed that the single-strand overhangs left by some restriction enzymes were better substrates for DNA ligase than blunt ends due to hydrogen bonding of the complementary single-stranded bases.



Substrates for DNA ligase are broken double helices. Blunt ends (top) or noncomplementary overhangs (center) are joined less efficiently than complementary overhangs (bottom). Also note the complementary overhangs in Figure 1-13.

Mung bean nuclease from Mung bean sprouts digests single-stranded DNA and RNA. Because it leaves double-stranded regions intact, it is used to remove overhangs from restriction fragments to produce blunt ends for cloning.

S1 nuclease from *Aspergillus oryzae* is another single-strand–specific nuclease. It hydrolyzes single-stranded DNA or RNA into 5′ mononucleotides. It also has endonuclease capability to hydrolyze single-stranded regions such as gaps and loops in duplex DNA. It was used extensively in early RNAse protection assays of gene expression. It is also used for nuclease mapping techniques.[24]

***recBC* nuclease** from *E. coli* is an ATP-dependent single- and double-stranded DNA nuclease. Although it has no activity at nicks (short single-strand gaps) in the DNA, it digests DNA from either the 3′ hydroxyl or the 5′ phosphate ends. It has some endonuclease activity on duplex DNA, generating short fragments, or **oligonucleotides**.

Micrococcal nuclease digests single- and double-stranded DNA and RNA at AT- or AU-rich regions. Although this enzyme can digest duplex DNA, it prefers single-stranded substrates. It is used in the laboratory to remove nucleic acid from crude extracts and also for analysis of chromatin structure.[25]

Deoxyribonuclease I (DNAse I) from bovine pancreas digests single-and double-stranded DNA at pyrimidines to oligodeoxyribonucleotides; so, technically, it is an endonuclease. It is used in both research and clinical laboratories to remove DNA from RNA preparations. DNAse I has also been used to detect exposed regions of DNA in DNA protein binding experiments.

DNA pol I from *E. coli* has exonuclease activity. Formerly called **exonuclease II**, this activity is responsible for the proofreading function of the polymerase.

As nucleases are natural components of cellular lysates, it is important to eliminate or inactivate them when preparing nucleic acid specimens for clinical analysis. Most DNA isolation procedures are designed to minimize both endonuclease and exonuclease activity during DNA isolation. Purified DNA is often stored in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to chelate cations required by nucleases for activity.

Helicases

DNA in bacteria and eukaryotes does not exist as the relaxed double helix as shown in Figure 1-1 but as a series of highly organized loops and coils. Release of DNA for transcription, replication, and recombination without tangling is brought about through cutting and reclosing of the DNA sugar-phosphate backbone. These functions are carried out by a series of enzymes called **helicases**.

As described with restriction endonucleases, the DNA double helix can be broken apart by the separation of the sugar phosphate backbones in both strands, a **double-strand break**. When only one backbone is broken (a **single-strand break** or nick), the broken ends are free to rotate around the intact strand. These ends can be digested by exonuclease activity or extended using the intact strand as a template (nick translation, as described above). The nicking and reclosing of DNA by helicases relieve topological stress in highly compacted, or **supertwisted**, DNA as required; for example, in advance of DNA replication or transcription. Helicases are of two types: **topoisomerases** and **gyrases**. Topoisomerases interconvert topological isomers or relax supertwisted DNA. Gyrases (**type II topoisomerases**) untangle DNA through double-strand breaks. They also separate linked rings of DNA (**concatamers**).

Topoisomerases in eukaryotes have activity similar to that in bacteria but with different mechanisms of cutting and binding to the released ends of the DNA. Because of their importance in cell replication, topoisomerases are the targets for several anticancer drugs, such as camptothecin, the epipodophyllotoxins VP-16 and VM-26, amsacrine, and the intercalating anthracycline derivatives doxorubicin and mitoxantrone. These **topoisomerase inhibitors** bring about cell death by interfering with the breaking and joining activities of the enzymes, in some cases trapping unfinished and broken intermediates.

Methyltransferases

DNA methyltransferases catalyze the addition of methyl groups to nitrogen bases, usually cytosine in DNA strands. Most prokaryotic DNA is methylated, or **hemimethylated** (methylated on one strand of the double helix and not the other), as a means to differentiate host DNA from nonhost and to provide resistance to restriction enzymes. Unlike prokaryotic DNA, eukaryotic DNA is methylated in specific regions. In eukaryotes, DNA binding proteins may limit accessibility or guide methyltransferases to specific regions of the DNA.

There are two main types of methyltransferases. **Maintenance methyltransferases** work throughout the life of the cell and methylate hemimethylated DNA. In contrast, **de novo methyltransferases** work only during embryonic development and may be responsible for the specific methylation patterns in differentiated cells.

Advanced Concepts

Enzymatic interconversion of DNA forms was first studied in vitro by observing the action of two *E. coli* enzymes, topoisomerase I[26] and gyrase[27] on circular plasmids. Topo I can relax supercoils in circular plasmid DNA by nicking one strand of the double helix. Gyrase, also called topoisomerase II, can introduce coiling by cutting both strands of the helix, passing another part of the duplex through and religating the cut strand.

Advanced Concepts

Both DNA and RNA helicases have been identified in molds, worms, and plants.[28–31] These enzymes may function in establishment of local chromosome architecture as well as regulation of transcription.

Advanced Concepts

Cytosine methyltransferases are key factors in vertebrate development and gene expression. These enzymes catalyze the transfer of an activated methyl group from **S-adenosyl methionine** to the 5 position of the cytosine ring (producing **5-methyl cytosine**). Methylation marks DNA for recognition by or resistance to enzymes such as nucleases or architectural proteins in higher eukaryotes. Methylation is the source of **imprinting** of DNA, a system that provides a predetermined program of gene expression during development. A specific methyl transferase, **Dmnt1**, prefers hemimethylated DNA, indicating a mechanism for keeping methylation patterns in the genome.[32] Defects in methylation have been observed in cancer cells, [33] some disease states,[34] and clones.[35] To date, three DNA cytosine methyltransferases have been cloned, DNMT1, DNMT3a, and DNMT3b.[36,37]

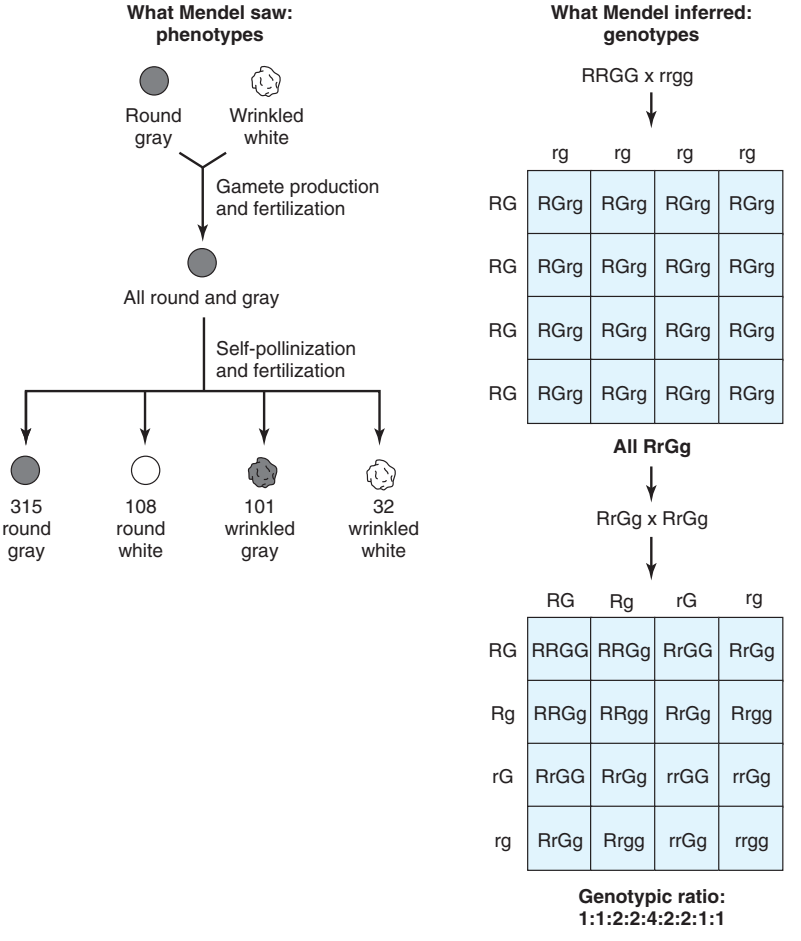
Recombination in Sexually Reproducing Organisms

Recombination is the mixture and assembly of new genetic combinations. Recombination occurs through the molecular process of crossing over or physical exchange between molecules. A **recombinant** molecule or organism is one that holds a new combination of DNA sequences

Based on Mendel's laws, each generation of sexually reproducing organisms is a new combination of the

Historical Highlights

Early studies of recombination were done with whole organisms. Mendel's analysis of peas (*Pisum* species)[38] established the general rules of recombination in sexually reproducing organisms. Mendel could infer the molecular exchange events that occurred in the plants by observing the phenotype of progeny. These observations had been made before, but by making quantitative predictions of the probability of phenotypes Mendel proposed that traits are inherited in a particulate manner, rather than blending as was previously thought.



■ Mendelian genetics showed that traits are inherited as unit characteristics. The probability of inheriting a given trait can then be calculated from the traits of the parents. (R-round, r-wrinkled, G-gray, g-white)

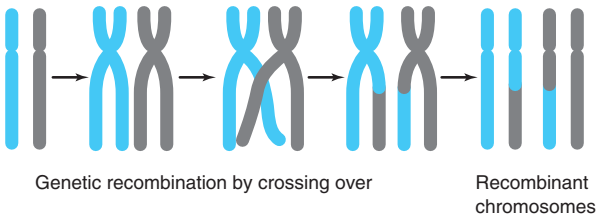


Figure 1-14 Generation of genetic diversity by crossing over of homologous chromosomes.

parental genomes. The mixing of genes generates genetic diversity, increasing the opportunity for more robust and well-adapted offspring. The beneficial effect of natural recombination is observed in **heteresis,** or hybrid vigor, observed in genetically mixed or hybrid individuals compared with purebred organisms.

Sexually reproducing organisms mix genes in three ways. First, at the beginning of **meiosis**, duplicated homologous chromosomes line up and recombine by **crossing over** or breakage and reunion of the four DNA duplexes (Fig. 1-14). This generates newly recombined duplexes with genes from each of the homologs. Then, these recombined duplexes are randomly assorted into gametes (Fig. 1-15), so that each gamete contains one set of each the recombined parental chromosomes. Finally, the gamete will merge with a gamete from the other parent carrying its own set of recombined chromosomes. The resulting offspring will contain a new set or recombination of genes of the parents. The nature of this recombination is manifested in the combinations of inherited traits of subsequent generations.

Recombinant DNA technology is a controlled mixing of genes. Rather than relying on natural mixing of whole genomes, single genes can be altered, replaced, deleted, or moved into new genomes. This directed diversity can produce organisms with predictable traits, as natural purebreds, but with single gene differences. The ability to manipulate single traits has implications not only in the laboratory but also in treatment and prevention of disease; for example, through gene therapy.

Recombination in Asexual Reproduction

Movement and manipulation of genes in the laboratory began with observations of natural recombination in asexually reproducing bacteria. Genetic information in asexually reproducing organisms can be recombined in

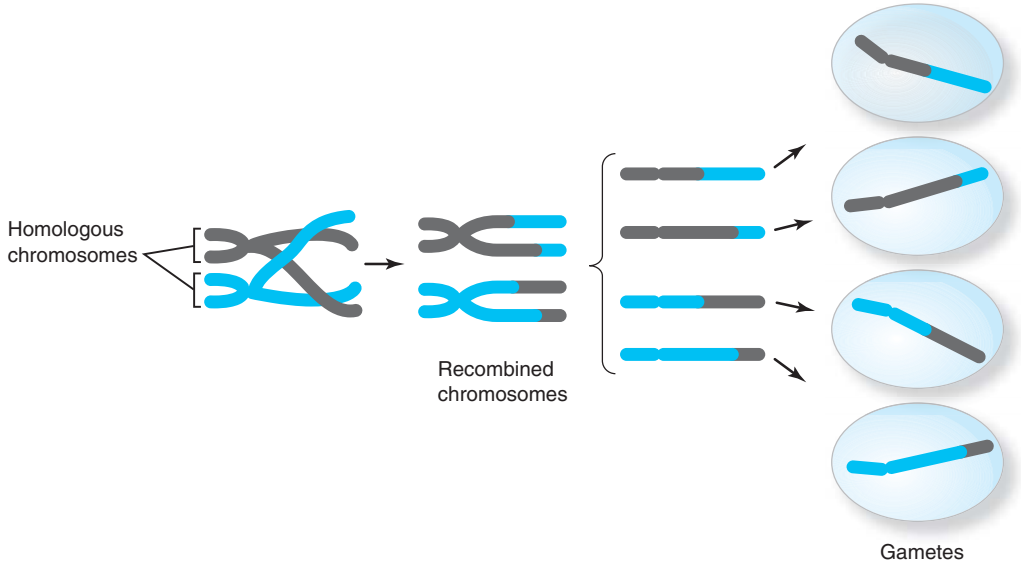
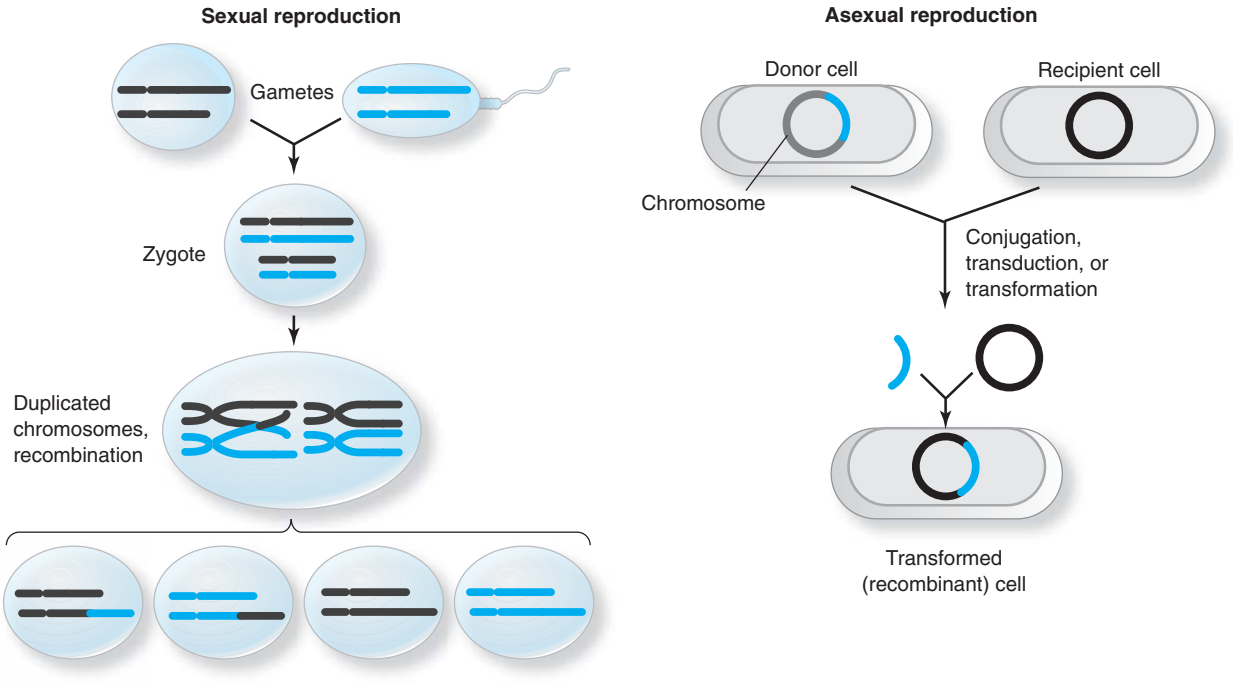


Figure 1-15 Recombined chromosomes are randomly assorted into gametes. Twenty-two other chromosomes will be randomly assorted into the four gametes, giving each one a new collection of recombined chromosomes.

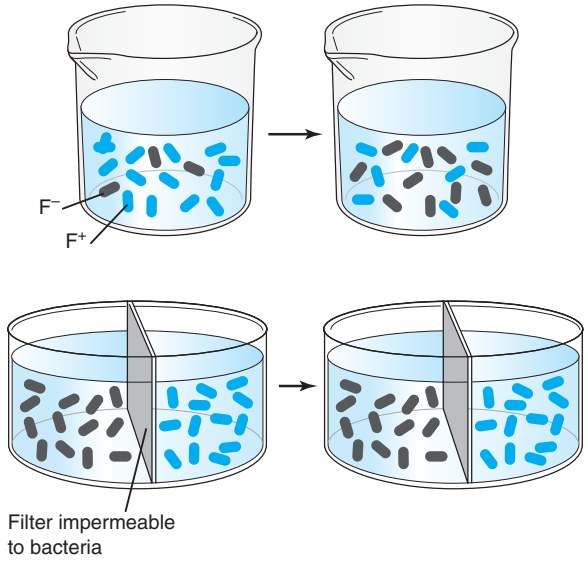


■ Figure 1-16 Recombination in sexual (left) and asexual (right) reproduction.

three ways: conjugation, transduction, and transformation (Fig. 1-16).

Conjugation

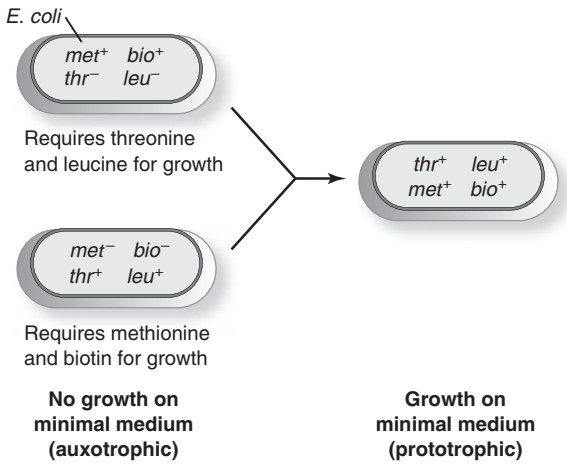
Bacteria that participate in **conjugation** are of two types, or sexes, termed **F+** and **F-**. For conjugation to occur, F- and F+ cells must be in contact with each other. Requirement for contact can be demonstrated by physically separating F+ and F- cells. If this is done, mating does not occur (Fig. 1-17). Microscopically, a filamentous bridge is observed between mating bacteria. Further work by J. Lederberg and William Hayes demonstrated **polarity** in the conjugation process; that is, genetic information could move from F+ to F- bacteria but not from F- to F+ bacteria. The explanation for this was soon discovered. The F+ bacteria had a "**fertility factor**" that not only carried the information from one cell to another but also was responsible for establishing the physical connection between the mating bacteria. The fertility factor was transferred from F+ to F- bacteria in the mating process, so that afterward the F- bacteria became F+ (Fig. 1-18).



■ Figure 1-17 Conjugating cells must be in physical contact with each other (top) for successful transfer of the F+ phenotype. If cells are separated by a membrane (bottom), F- bacteria do not become F+.

Historical Highlights

Historically, recombination was studied through controlled mating and propagation of organisms. George Beadle[39] and others confirmed the connection between the units of heredity and physical phenotype using molds (*Neurospera crassa*), bacteria, and viruses. Joshua Lederberg and Edward L. Tatum[40] demonstrated that bacteria mate and exchange genetic information to produce recombinant offspring. Lederberg and Tatum proved that genetic exchange between organisms was not restricted to the sexually reproducing molds. These early studies first demonstrated the existence of recombination in *E. coli*.



Transfer of genetic information by conjugation can be demonstrated using double mutants. Bacteria with double mutations that required exogenous methionine and biotin (*met* and *bio*) or threonine and leucine (*thr* and *leu*) cannot grow on a medium without addition of these nutrients (minimal medium). When these strains are mixed together, however, growth occurs. The resulting bacteria have acquired the normal genes (+) through transfer or conjugation.

The F factor was subsequently shown to be an extrachromosomal circle of double-stranded DNA carrying the genes coding for construction of the mating bridge. Genes carried on the F factor are transferred across the bridge and simultaneously replicated, so that one copy of the F factor remains in the F+ bacteria, and the other is introduced into the F- bacteria. After mating, both bacteria are F+. The F factor may be lost or cured during normal cell division, turning an F+ bacteria back to the F− state.

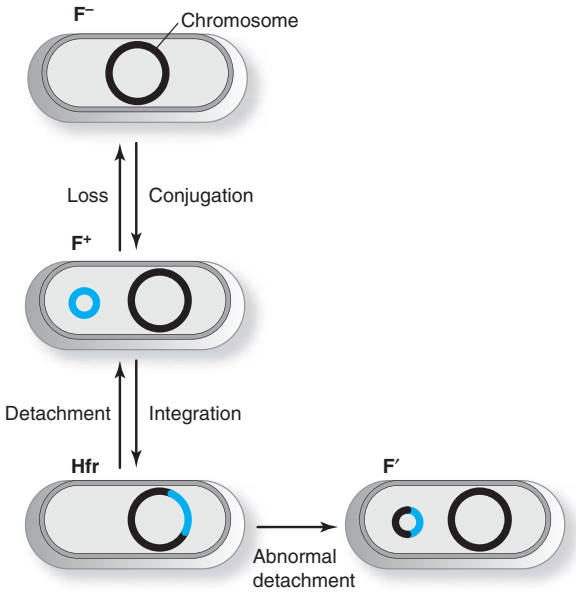


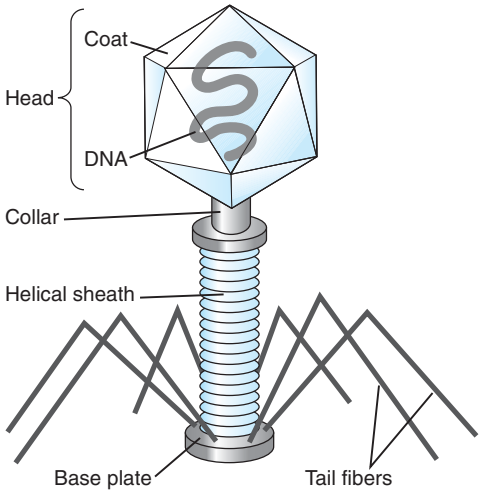
Figure 1-18 Fertility (the ability to donate genetic information) is controlled by the F factor (green). The F factor can exist by itself or be integrated into the host chromosome (large black circle).

The F factor can also insert itself into the host chromosome through a crossover or recombination event. Embedded in the chromosome, the F factor maintains its ability to direct mating and can carry part or all of the host chromosome with it across the mating bridge into the F- bacteria. Strains with chromosomally embedded F factors are called **Hfr** bacteria, for high frequency of recombination. The embedded F factor in these rarely occurring strains pulls host chromosomal information into recipient bacteria where another recombination event can insert that information into the recipient chromosome, forming a recombinant or new combination of genes of the Hfr and F- bacteria. Hfr bacteria were used in the first mapping studies.

Transduction

In the early 1960s, Francois Jacob and Elie Wollman[41] studied the transmission of units of heredity carried by

viruses from one bacterium to another (**transduction**). Just as animal and plant viruses infect eukaryotic cells, bacterial viruses, or **bacteriophages**, infect bacterial cells. The structure of bacteriophage T4 is one example of the specialized protein coats that enable these viruses to insert their DNA through the cell wall into the bacterial cell (Fig. 1-19). Alfred Hershey and Martha Chase confirmed that the DNA of a bacterial virus was the carrier of its genetic determination in the transduction process.[42] Hershey and Chase used ^{35}S to label the viral protein and ^{32}P to label the viral DNA. The experiment showed that viral protein remained outside of the cell while viral DNA entered the cell. Furthermore, ^{32}P-labeled DNA could be detected in new viruses generated in the transduction process (Fig. 1-20). Methods soon developed using bacteriophages to move genetic information between bacteria by growing the phage on one strain of bacteria and then infecting a second strain with those viruses. Transduction is also useful in determining gene order. Seymour Benzer used transduction of the T4 bacterial virus to fine-map genes.[43]

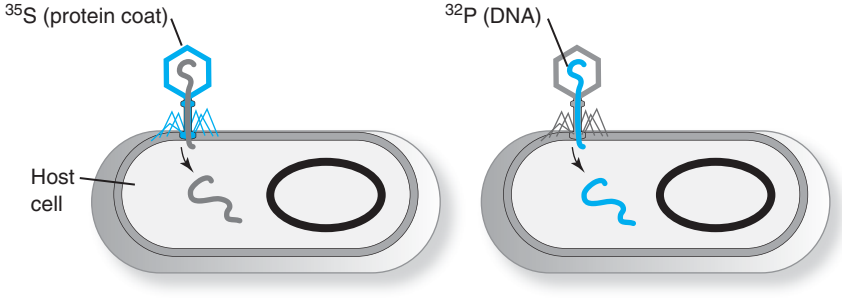


■ Figure 1-19 Bacteriophage T4 infects specific strains of *E. coli*. It has specialized structures. The tail fibers find the bacterial surface and allow contact of the tail plate and injection of the DNA in the viral head through the sheath into the bacterium.

Transformation

Although conjugation and transduction were the methods for the initial study of the connection between DNA and phenotype, **transformation**, which had first been observed in 1928 by Frederick Griffith,[44] is the basis for modern-day recombinant techniques. Griffith was investigating virulence in *Diplococcus* (now known as *Streptococcus*) *pneumoniae*. He had two strains of the bacteria: one with a rough colony type that was avirulent and one with a smooth colony type that was virulent. Griffith intended to use these strains to develop a protective vaccine (Fig. 1-21). He knew that the live smooth-type bacteria were lethal in mice, and the live rough-type were not. If he first killed the smooth-type bacteria by boiling, virulence was lost, and they were no longer lethal to mice. Surprisingly, when he mixed killed smooth-type and live rough-type bacteria, virulence returned. Furthermore, he could recover live smooth-type bacteria from the dead mice. He concluded that something from the dead smooth-type bacteria had "transformed" the rough-type bacteria into smooth-type.

What Griffith had observed was the transfer of DNA from one organism to another without the protection of a conjugative bridge or a viral coat. Fifteen years later, Oswald T. Avery, Colin MacLeod, and M.J. McCarty identified the transforming material as DNA.[45,46] They prepared boiled virulent bacterial cell lysates and sequen-



■ Figure 1-20 Radioactive (green) protein does not enter the host cell during transduction (left). Radioactive DNA, however, does enter (right) and is passed to subsequent generations of viruses.

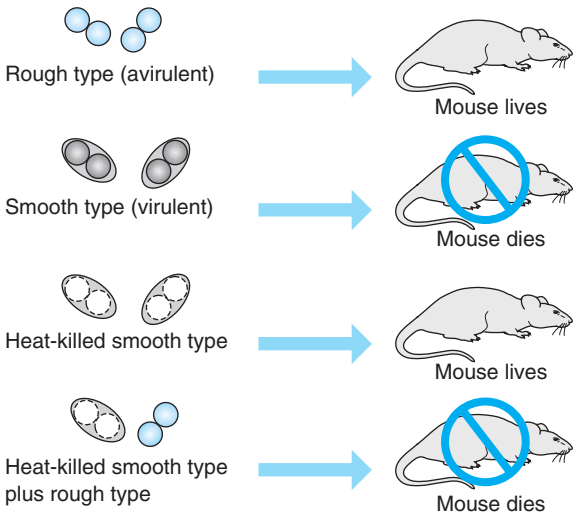


Figure 1-21 The "transforming factor" discovered by Griffith was responsible for changing the phenotype of the avirulent rough type bacteria to that of the virulent smooth type.

tially treated them with recently discovered enzymes (Fig. 1-22). **Protease** and **ribonuclease** treatment, which degraded protein and RNA, respectively, did not affect the transformation phenomenon that Griffith had demonstrated earlier. Treatment with deoxyribonuclease, which degrades DNA, however, prevented transformation. They concluded that the "**transforming factor**" that Griffith had first proposed was DNA. The transduction experiment of Alfred Hershey and Martha Chase also confirmed their findings that DNA carried genetic traits.

Advanced Concepts

Investigators performing early transformation studies observed the transfer of broken chromosomal DNA from one population of bacterial cells to another. Naked DNA transferred in this way, however, is a very inefficient source for transformation. Unprotected DNA is subject to physical shearing as well as chemical degradation from naturally occurring nucleases, especially on the broken ends of the DNA molecules. Natural transformations are much more efficient, because the transforming DNA is in circular form.

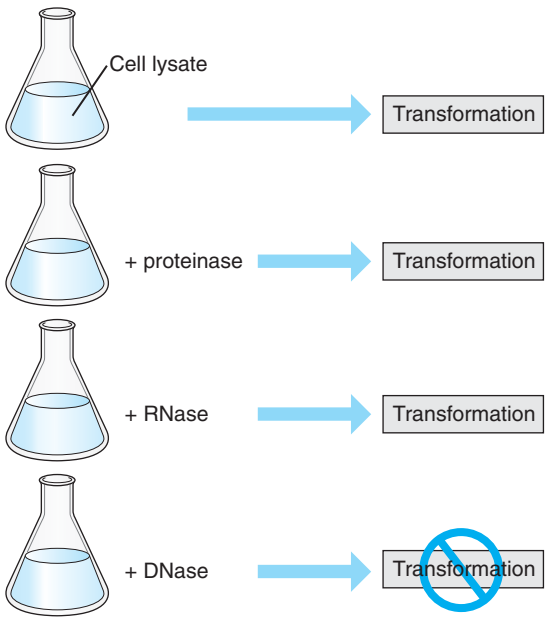


Figure 1-22 Avery, MacLeod, and McCarty showed that destruction of protein or RNA in the cell lysate did not affect the transforming factor. Only destruction of DNA prevented transformation.

Plasmids

DNA helices can assume both linear and circular forms. Most bacterial chromosomes are in circular form. Chromosomes in higher organisms, such as fungi, plants, and animals, are mostly linear. The ends of linear chromosomes are protected by specialized structures called **telomeres**. A cell can contain, in addition to its own chromosome complement, extrachromosomal entities, or **plasmids** (Fig. 1-23). Most plasmids are double-stranded circles, 2000–100,000 bp (2–100 kilobase pairs) in size. Just as chromosomes do, plasmids carry genetic information. Due to their size and effect on the host cell, plasmids can carry only a limited amount of information. The plasmid DNA duplex is compacted, or **supercoiled**. Breaking one strand of the plasmid duplex, or nicking, will relax the supercoil (Fig. 1-24), whereas breaking both strands will linearize the plasmid. Different physical states of the plasmid DNA can be resolved by distinct migration characteristics during gel electrophoresis.

Plasmids were discovered to be the source of resistant phenotypes in multidrug-resistant bacteria.[47] The demon-

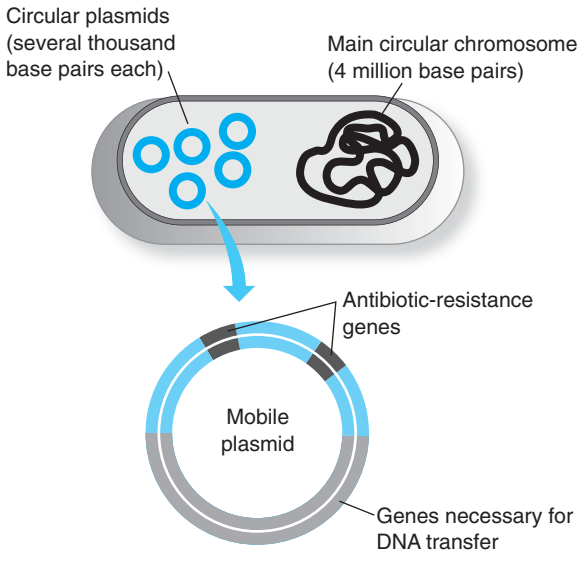


Figure 1-23 Plasmids are small extrachromosomal DNA duplexes that can carry genetic information.

stration that multiple drug resistance in bacteria can be eliminated by treatment with acridine dyes[48] was the first indication of the **episomal** (plasmid) nature of the resistance factor, similar to the F factor in conjugation. The plasmids, which carry genes for inactivation or circumvention of antibiotic action, were called resistance transfer factors (RTF), or **R factors**. R factors carry resistance to common antibiotics such as chloramphenicol, tetracycline, ampicillin, and streptomycin. Another class of plasmid, **colicinogenic factors**, carries resistance to **bacteriocins**, toxic proteins manufactured by bacteria. Plasmids can replicate in the host cell but cannot survive outside of the cell as viruses do. The acquisition of the resistance genes from host chromosomes of unknown bacteria is the presumed origin of these resistance factors.[49] Drug-resistance genes are commonly gained and lost from episomes in a bacterial population. Simultaneous introduction of R factors into a single cell can result in recombination between them, producing a new, recombinant plasmid with a new combination of resistance genes.

Plasmids were initially classified into two general types: **large plasmids** and **small plasmids**. Large plasmids include the F factor and some of the R plasmids. Large plasmids carry genes for their own transfer and propagation and are **self-transmissible**. Large plasmids occur in small numbers, one or two copies per chromosome equivalent. Small plasmids are more numerous in the cell, about 20 copies per chromosomal equivalent; however, they do not carry genes directing their maintenance. They rely on high numbers for distribution into daughter cells at cell division or uptake by host cells in transformation.

Compared with fragments of DNA, plasmids are more efficient vehicles for the transfer of genes from one cell to another. Upon cell lysis, supercoiled plasmids can enter other cells more efficiently. Plasmids have been used extensively in recombinant DNA technology to introduce specific traits. By manipulation of the plasmid DNA in vitro, specific genes can be introduced into cells

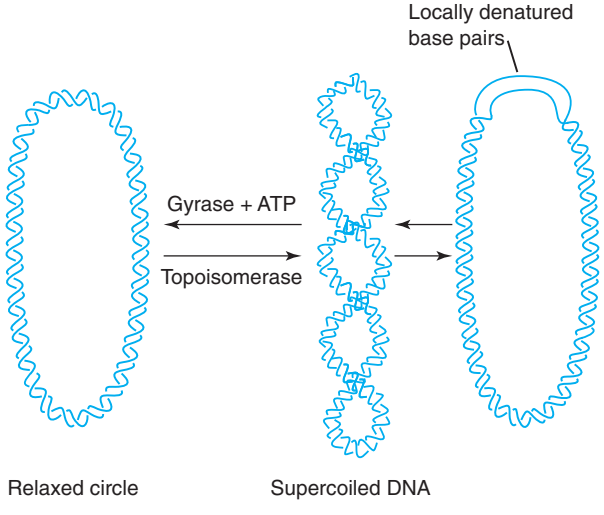


Figure 1-24 Supercoiled plasmids can be relaxed by nicking (left) or by local unwinding of the double helix (right).

Advanced Concepts

The circular nature of R factors was demonstrated by buoyant density centrifugation.[50] Plasmid DNA has a density higher than that of the host chromosome and can be isolated from separate, or **satellite**, bands in the gradient. Examination of the fractions of the higher density DNA revealed small circular species. These circles were absent from drug-sensitive bacteria.

Advanced Concepts

Plasmids are found not only in bacteria but in multicellular plants and animals as well. Some viruses, such as the single-stranded DNA virus M13, have a transient plasmid phase in their life cycle. Laboratory techniques requiring single-stranded versions of specific DNA sequences have been based on the manipulation of the plasmid (duplex circle) phase of these viruses and isolation of the single-stranded recombinant single-stranded circles from the virus. This technology was used in methods devised to determine the order or sequence of nucleotides in the DNA chain.

to produce new phenotypes or recombinant organisms. The ability to express genetic traits from plasmids makes it possible to manipulate phenotype in specific ways. As will be described in later chapters, plasmids play a key role in the development of procedures used in molecular analysis.

• STUDY QUESTIONS •

DNA Structure and Function

1. What is the function of DNA in the cell?
2. Compare the structure of the nitrogen bases. How do purines and pyrimidines differ?
3. Write the complementary sequence to the following: 5′AGGTCACGTCTAGCTAGCTAGA3′
4. Which of the ribose carbons participate in the phosphodiester bond?
5. Which of the ribose carbons carries the nitrogen base?
6. Why does DNA polymerase require a primer?

Restriction Enzyme Analysis

1. A plasmid was digested with the enzyme, *Hpa*II. On agarose gel electrophoresis, you observe three bands, 100, 230, and 500 bp.
   a. How many *Hpa*II sites are present in this plasmid?
   b. What are the distances between each site?
   c. What is the size of the plasmid?
   d. Draw a picture of the plasmid with the *Hpa* II sites.

   A second cut of the plasmid with *Bam*H1 yields two pieces, 80 and x bp.
   e. How many *Bam*H1 sites are in the plasmid?
   f. What is x in base pairs (bp)?
2. How would you determine where the *Bam*H1 sites are in relation to the *Hpa*II sites?
3. The plasmid has one *Eco*R1 site into which you want to clone a blunt-ended fragment. What type of enzyme could turn an *Eco*R1 sticky end into a blunt end?

Recombination and DNA Transfer

1. Compare how DNA moves from cell to cell by a) conjugation, b) transduction, and c) transformation.

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Chapter 2 *Lela Buckingham*

RNA

OUTLINE

TRANSCRIPTION

TYPES/STRUCTURES

Ribosomal RNA
Messenger RNA
Small Nuclear RNA
Small Interfering RNA
Transfer RNA
Micro RNAs
Other Small RNAs

RNA POLYMERASES

OTHER RNA-METABOLIZING ENZYMES

Ribonucleases
RNA Helicases

REGULATION OF TRANSCRIPTION

Epigenetics

OBJECTIVES

- Compare and contrast the structure of RNA with that of DNA.
- List and compare the different types of RNA.
- Describe the cellular processing of messenger RNA.
- List several types of RNA polymerases, their substrates and products.
- Recognize the reactions catalyzed by ribonucleases and RNA helicases and their roles in RNA metabolism.
- Describe how ribonucleotides are polymerized into RNA (transcription) and the relation of the sequence of the RNA transcript to the DNA sequence of its gene.
- Describe gene regulation using the Lac operon as an example.
- Define epigenetics and list examples of epigenetic phenomena.

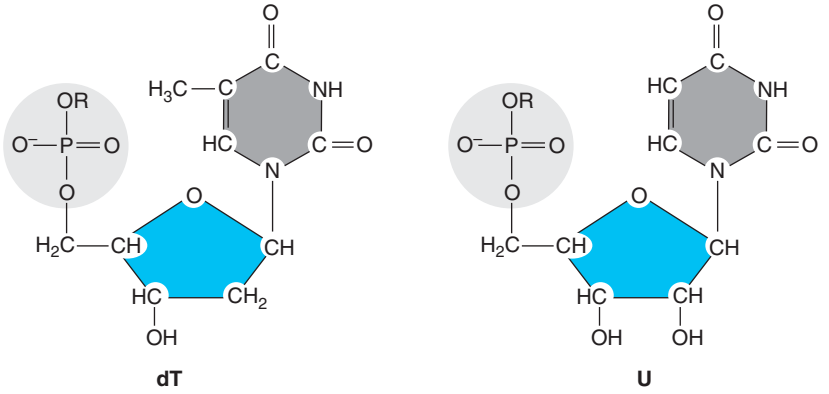


Figure 2-1 Uracil (U), the nucleotide base that replaces thymine in RNA, has the purine ring structure of thymine (dT) minus the methyl group. Uracil forms hydrogen bonds with adenine.

Ribonucleic acid (RNA) is a polymer of nucleotides similar to DNA. It differs from DNA in the sugar moieties, having **ribose** instead of deoxyribose and, in one nitrogen base component, having **uracil** instead of thymine (thymine is 5-methyl uracil; Fig. 2-1). Furthermore, RNA is synthesized as a single strand rather than as a double helix. Although RNA strands do not have complementary partner strands, they are not completely single-stranded. Through internal homologies, RNA species fold and loop upon themselves to take on as much of a double-stranded character as possible. RNA can also pair with complementary single strands of DNA or RNA and form a double helix.

There are several types of RNAs found in the cell. Ribosomal RNA, messenger RNA, transfer RNA, and small nuclear RNAs have distinct cellular functions. RNA is copied, or **transcribed**, from DNA.

Advanced Concepts

Evolutionary theory places RNA as the original genetic material from which DNA has evolved. In most organisms, RNA is an intermediate between the storage system of DNA and the proteins responsible for phenotype. One family of RNA viruses, the **retroviruses**, which include leukemia viruses and the human immunodeficiency virus, have RNA genomes and, in order to replicate using host cell machinery, must first make a DNA copy of their genome by **reverse transcription**.

Transcription

DNA can only store information. In order for this information to be utilized, it must be transcribed and then translated into protein, a process called **gene expression**. A specific type of RNA, messenger RNA (mRNA), carries the information in DNA to the ribosomes where it is translated into protein.

Transcription is the copying of one strand of DNA into RNA by a process similar to that of DNA replication. This activity occurs mostly in interphase nuclei. Evidence[1] suggests that transcription takes place at discreet stations of the nucleus into which the DNA molecules move. One of these sites, the **nucleolus,** is the location of ribosomal RNA synthesis.

The polymerization of RNA from a DNA template is catalyzed by RNA polymerase. After binding to its start site in DNA, a specific sequence of bases called the **promoter**, RNA polymerase and its supporting accessory proteins synthesize RNA using the base sequence of one strand of the double helix (the **antisense strand**) as a

Advanced Concepts

DNA must be released locally from histones and the helix unwound in order for transcription to occur. These processes involve the participation of numerous factors, including DNA binding proteins, transcription factors, histone modification enzymes, and RNA polymerase.

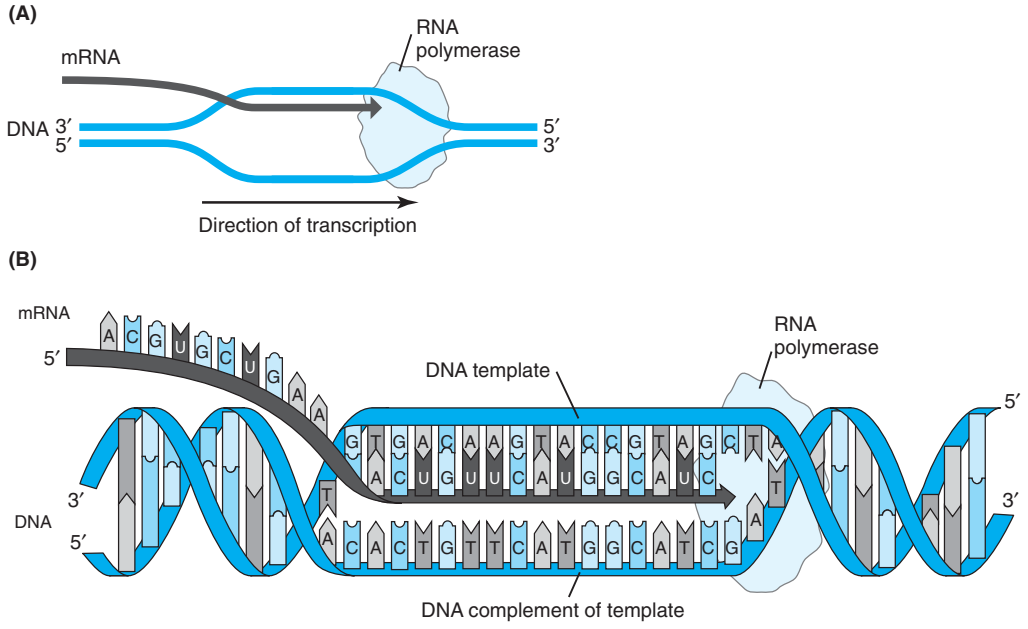


Figure 2-2 RNA polymerase uses one strand of the double helix (the antisense strand) as a template for synthesis of RNA. About 10 base pairs of DNA are unwound or opened to allow the polymerase to work.

guide (Fig. 2-2). The **sense strand** of the DNA template has a sequence identical to that of the RNA product (except for the U for T substitution in RNA), but it does not serve as the template for the RNA.

Compared with sites of initiation of DNA replication, there are many more sites for initiation of transcription (RNA synthesis) in both prokaryotes and eukaryotes. There are also many more molecules of RNA polymerase than DNA polymerase in the cell. RNA polymerases work more slowly than DNA polymerases (50–100 bases/sec for RNA synthesis vs. 1000 bases/sec for DNA replication) and with less fidelity.

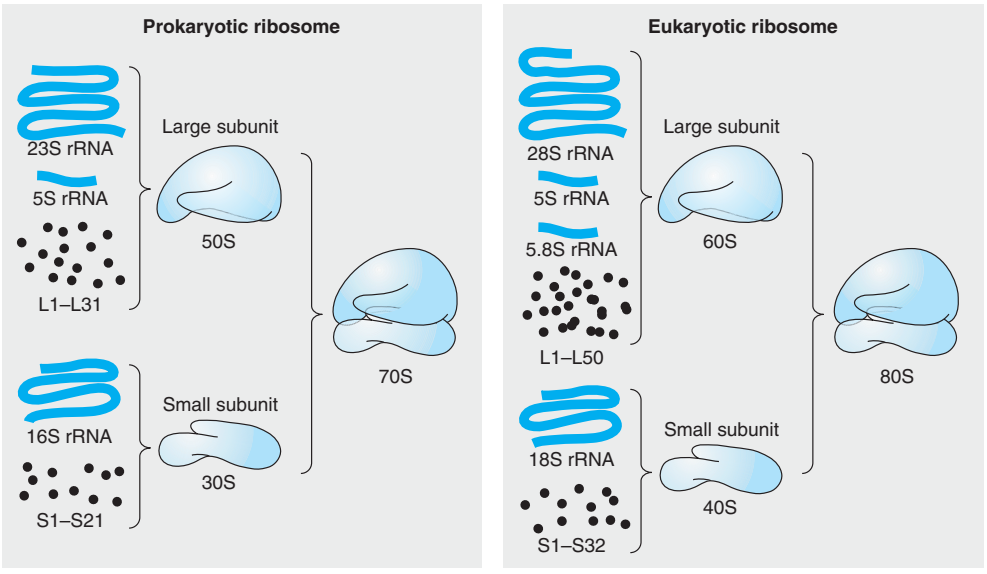
Unlike DNA synthesis, RNA synthesis does not require a primer. Upon initiation of RNA synthesis, the first ribonucleoside triphosphate retains all of its phosphate groups as the RNA is polymerized in the 5′ to 3′ direction. Subsequent ribonucleoside triphosphates retain only the **alpha phosphate**, the one closest to the ribose sugar. The other two phosphate groups are released as orthophosphate during the synthesis reaction.

RNA synthesis proceeds along the DNA template until the polyadenylation signal is encountered. At this point the process of termination of transcription is activated. There is no consensus sequence in DNA that specifies termination of transcription. This might be explained by recent descriptions of termination by specific exonuclease activity.[2] As the polymerase proceeds past the polyA site, the nascent mRNA is released by an endonuclease associated with the carboxy terminal end of the polymerase. RNA synthesized beyond the site trails out of the RNA polymerase and is bound by another exonuclease that begins to degrade the RNA 5′ to 3′ toward the RNA polymerase. When the exonuclease catches up with the polymerase, transcription stops.

Gene expression is the fundamental process for cell regulation, differentiation, and development. Signal transduction pathways that are the targets of several therapeutic strategies funnel internal and external signals to the nucleus where **transcription factors** bind to specific sequences in DNA and initiate or turn off transcription.

Types/Structures

There are several types of RNAs found in the cell. Ribosomal RNA, mRNA, transfer RNA, and small nuclear RNAs have distinct cellular functions.



■ Figure 2-3 Prokaryote and eukaryote ribosomal subunits are of similar structure but different size. Ribosomal RNAs (left) are assembled with 52 or 82 ribosomal proteins (center) to make the subunits that will form the complete ribosome in association with mRNA.

Ribosomal RNA

The largest component of cellular RNA is **ribosomal RNA** (rRNA), comprising 80%-90% of the total cellular RNA. The various types of ribosomal RNAs are named for their **sedimentation coefficient** (S) in density gradient centrifugation.[3] rRNA is an important structural and functional part of the ribosomes, cellular organelles where proteins are synthesized (Fig. 2-3).

In prokaryotes, there are three rRNA species, the **16S** found in the ribosome small subunit, and the **23S** and **5S**, found in the ribosome large subunit, all synthesized from the same gene. In eukaryotes, rRNA is synthesized from highly repeated gene clusters. Eukaryotic rRNA is copied from DNA as a single 45S precursor RNA (**preribosomal RNA**) that is subsequently processed into **18S** of the ribosome small subunit and **5.8S** and **28S** species of the large subunit. Another rRNA species, 5S, found in the large ribosome subunit in eukaryotes, is synthesized separately.

Messenger RNA

Messenger RNA (mRNA) is the initial connection between the information stored in DNA and the translation apparatus that will ultimately produce the protein products responsible for the phenotype dictated by the chromosome. In prokaryotes, mRNA is synthesized even as it is being translated into protein. Prokaryotic mRNA is sometimes **polycistronic**; that is, coding for more than one protein on the same mRNA. Eukaryotic mRNA, in contrast, is monocistronic, having only one protein per mRNA. Eukaryotes can, however, produce different proteins from the same DNA sequences by starting the RNA synthesis in different places or by processing the mRNA differently. In eukaryotes, copying of RNA from DNA and protein synthesis from the RNA are separated by the nuclear membrane barrier. Eukaryotic mRNA undergoes a series of **post-transcriptional processing** events before it is translated into protein (Fig. 2-4).

Advanced Concepts

The secondary structure of rRNA is important for the integrity and function of the ribosome. Not only is it important for ribosomal structure, it is also involved in the correct positioning of the ribosome on the mRNA and with the transfer RNA during protein synthesis.[50, 51]

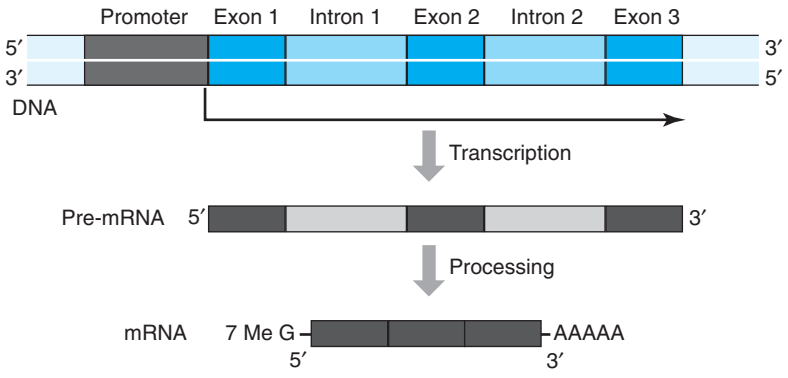


Figure 2-4 DNA (top) and heteronuclear RNA (middle) contain intervening (intron) and expressed (exon) sequences. The introns are removed and the mature RNA is capped and polyadenylated during processing (bottom).

The amount of a particular mRNA in a cell is related to the requirement for its final product. Some messages are transcribed constantly and are relatively abundant in the cell (**constitutive** transcription), whereas others are transcribed only at certain times during the cell cycle or under particular conditions (**inducible**, or regulatory, transcription). Even the most abundantly transcribed mRNAs are much less plentiful in the cell than rRNA.

Messenger RNA Processing

Polyadenylation

Study of mRNA in eukaryotes was facilitated by the discovery that most messengers carry a sequence of polyadenylic acid at the 3′ terminus, the **poly(A) tail**. The run of adenines was first discovered by hydrogen bonding of mRNA to polydeoxythymine on poly(dT) cellulose.[4] Polyuridine or polythymine residues covalently attached to cellulose or sepharose substrates are often used to specifically isolate mRNA in the laboratory.

The poly(A) tail is not coded in genomic DNA. It is added to the RNA after synthesis of the pre-mRNA. A protein complex recognizes the RNA sequence, AAUAAA, and cleaves the RNA chain 11–30 bases 3′ to that site. The enzyme that cuts pre-mRNA in advance of polyadenylation has not been identified. Recent studies suggest that a component of the protein complex related to the system that is responsible for removing the 3′ extension from pre-transfer RNAs may also be involved in generation of the 3′ ends on mRNA.[5,6] The enzyme **polyadenylate polymerase** is responsible for adding the adenines to the end of the transcript. A run of up to 200 nucleotides of poly(A) is typically found on mRNA in mammalian cells.

Historical Highlights

A DNA copy (complementary DNA, copy DNA [**cDNA**]) of mature mRNA can be made by reverse transcription of mRNA (synthesis of DNA from the mRNA template). Compared with the original gene on the chromosome, the cDNA version of eukaryotic genes is smaller than the chromosomal version. Restriction enzyme mapping can be used to confirm that this is not due to premature termination of the genomic transcript. The entire functional gene is present in the shorter sequence because cDNA versions of eukaryotic genes can be expressed (transcribed and translated) into complete proteins.[8] The larger chromosomal version of the gene must, therefore, have extra sequences, and these sequences must be inserted between the protein coding sequences. Direct location and size of these intervening sequences were first demonstrated by electron microscopy of hybrids between mRNA and cDNA[69] using the method of **R loop mapping** developed by White and Hogness.[70] In these experiments, mRNA and duplex genomic DNA were incubated together at elevated temperatures in a high concentration of formamide. Under these conditions, the more stable RNA-DNA hybrids are favored over the DNA duplexes. The resulting structures released loops of unpaired DNA (introns) that could be measured. The DNA duplexed with RNA corresponded to the coding sequences (exons).

Advanced Concepts

About 30% of the mRNAs, notably histone mRNAs, are not polyadenylated.[52] The function of the poly(A) tail, or functional differences between poly(A)+ and poly(A)- mRNA, is not clear. The polyA tail may be involved in movement of the mRNA from the nucleus to the cytoplasm, association with other cell components, maintenance of secondary structure, or proper stability of the message.

Capping

Eukaryotic mRNA is blocked at the 5′ terminus by an unusual 5′-5′ pyrophosphate bridge to a methylated guanosine.[7] The structure is called a **cap**. The cap is a 5′-5′ pyrophosphate linkage of 7-methyl guanosine to either 2′ O-methyl guanine or 2′ O-methyl adenine of the mRNA,

$$^{7\text{-methyl}}G^{5'}ppp\ ^{5'}\ G \text{ or } A\ ^{2'\text{O-methyl}}\ pNpNpNp$$

where p represents a phosphate group, N represents any nucleotide.

The cap confers a protective function as well as serves as a recognition signal for the translational apparatus. Caps differ with respect to the methylation of the end nucleotide of the mRNA. In some cases, 2′O-methylation occurs not only on the first but also on the second nucleotide from the cap. Other caps methylate the first three nucleotides of the RNA molecule.

Splicing

Prokaryotic structural genes contain uninterrupted lengths of **open reading frame**, sequences that code for amino acids. In contrast, eukaryotic coding regions are interrupted with long stretches of noncoding DNA sequences called **introns**. Newly transcribed mRNA, **heteronuclear RNA** (hnRNA), is much larger than mature mRNA because it still contains the intervening sequences. Labeling studies demonstrated that the hnRNA is capped and tailed and that these modifications survive the transition from hnRNA to mRNA, which is simply a process of removing the **intervening sequences** from the hnRNA.

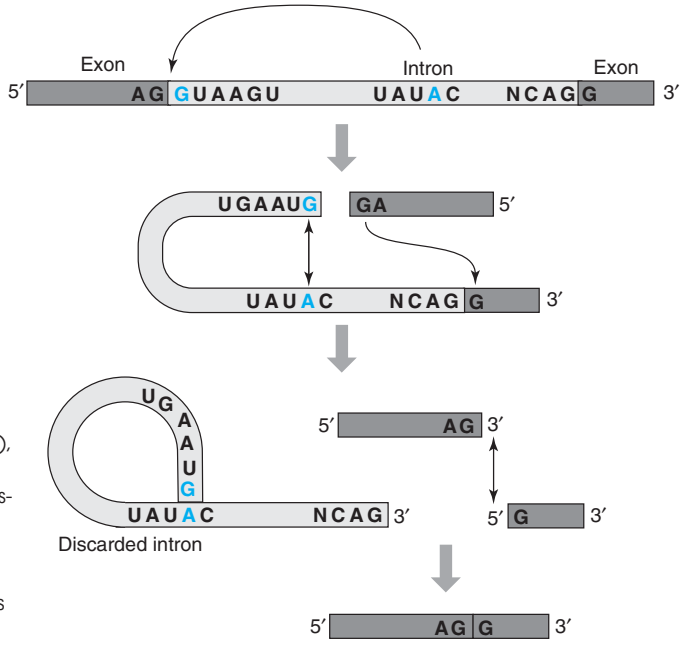
Introns are removed from hnRNA by **splicing** (Fig. 2-5). The remaining sequences that code for the protein product are **exons**.

Advanced Concepts

Caps are present on all eukaryotic mRNA bound for translation, except for some mRNA transcribed from mitochondrial DNA. Capping occurs after initiation of transcription, catalyzed by the enzyme **guanylyl transferase**. This enzyme links a guanosine monophosphate provided by guanosine triphosphate to the 5′ phosphate terminus of the RNA with the release of pyrophosphate. In some viruses, guanosine diphosphate provides the guanidine residue, and monophosphate is released. Caps of mRNA are recognized by ribosomes just before translation.[53]

Advanced Concepts

There are four types of introns, **group I, group II, nuclear,** and **tRNA,** depending on the mechanism of their removal from hnRNA. Group I introns are found in nuclear, mitochondrial, and chloroplast genes. Group II introns are found in mitochondrial and chloroplast genes. Group I introns require a **guanosine triphosphate** molecule to make a nucleophilic attack on the 5′ phosphate of the 5′ end of the intron. This leaves a 3′ OH at the end of the 5′ exon (**splice donor site**), which attacks the 5′ end of the next exon (**splice acceptor site**), forming a new phosphodiester bond and releasing the intervening sequence. Group II introns are removed in a similar reaction initiated by the 2′ OH of an adenosine within the intron attacking the 5′ phosphate at the splice donor site. When the 3′ OH of the splice donor site bonds with the splice acceptor site of the next exon, the intervening sequence is released as a **lariat** structure (see Fig. 2-4). This lariat contains an unusual 2′, 3′, and 5′ triply-linked nucleotide, the presence of which proved the mechanism. Removal of nuclear introns occurs by the same transesterification mechanism, except this reaction is catalyzed by specialized RNA-protein complexes (**small nucleoprotein particles**). These complexes contain the **small nuclear RNAs** U1, U2, U4, U5, and U6.



■ Figure 2-5 RNA splicing at the 5′ splice site (AGGUAAGU), branch (UAUAC), and 3′ splice site (NCAGG) consensus sequences. The intron (light gray) is removed through a transesterification reaction involving a guanine nucleotide of the 5′ site and an adenine in the branch sequence. The product of this reaction is the discarded intron in a lariat structure. Another transesterification reaction connects the exons.

Although removal of all nuclear introns requires protein catalysts, some introns are removed without the participation of protein factors in a **self-splicing** reaction. The discovery of self-splicing was the first demonstration that RNA could act as an enzyme.

Inspection of splice junctions from several organisms and genes has demonstrated the following consensus sequences for the donor and acceptor splice junctions of group I, II, and nuclear introns[8]:

A G // G U A A G U	(intron)	Y N C U R A C	Y_N N C A G // G
splice		branch point	splice
donor site		sequence	acceptor site

Advanced Concepts

The splicing of transfer RNA (tRNA) transcripts involves breakage and reunion of the RNA chain. Endonucleases cleave the tRNA precisely at the intron ends. The resulting tRNA ends, a 2′, 3′ cyclic phosphate and a 5′ OH, are then ligated in a complex reaction that requires ATP, followed by further base modification in some tRNAs.

The branch point sequence YNCURAC is variable in mammals but almost invariant in the yeast *Saccharomyces cervisiae* (UACUAAC).

Splicing may be important for timing of translation of mRNA in the cytoplasm, although it is not necessarily required as cloned genes synthesized in vitro without introns are expressed in eukaryotic cells. Introns may have evolved as a means of increasing recombination frequency within genes as well as between genes.[8] The discontinuous nature of eukaryotic genes may also protect the coding regions from genetic damage by toxins or radiation.

Alternative splicing can modify products of genes by alternate insertion of different exons. For example, the production of calcitonin in the thyroid or calcitonin gene-related peptide in the brain depends on the exons included in the mature mRNA in these tissues.[9] Alternative splicing has been found in about 40 different genes.

Abnormalities in the splicing process are responsible for several disease states. Some β-thalassemias result from mutations in splice recognition sequences of the β-globin genes. Certain autoimmune conditions result from production of antibodies to RNA protein complexes. Auto-antibodies against U1 RNA, one of the small nuclear RNAs required for splicing, are associated with systemic lupus erythematosus.

Small Nuclear RNA

Another type of cellular RNA is the **small nuclear RNA** (snRNA), which functions in splicing (removal of introns from freshly transcribed RNA) in eukaryotes. Small nuclear RNA stays in the nucleus after its transcription by RNA polymerase I or III. Small nuclear RNAs from eukaryotic cells sediment in a range of 6-8S. Small nuclear RNAs isolated from hepatoma and cervical carcinoma cell lines are summarized in Table 2.1.

Small nuclear RNAs serve mostly a structural role in the processing of mRNA. Several of a family of proteins (**Sm proteins**) assemble into a (60Å by 30-40Å) doughnut-shaped complex that interacts with the U-rich regions of poly (U) RNAs.[10,11]

U1 RNA is complementary to sequences at the splice donor site, and its binding distinguishes the sequence GU in the splice site from other GU sequences in the RNA. U2 RNA recognizes the splice acceptor site. In lower eukaryotes, another protein, splicing factor 1,[12] binds to the branch point sequence, initiating further protein assembly and association of U4, U5, and U6, with the looped RNA forming a complex called the **splicesosome** in which the transesterification reaction linking the exons together takes place.

Table 2.1 **Small Nuclear RNA Isolated From HeLa Cervical Carcinoma and Novikoff Hepatoma Cells[8]**

Species (HeLa)	Species (Novikoff)	Approximate Length (Bases)
SnA	U5	180
SnB		210
SnC	U2	196
SnD	U1B	171
SnE/ScE (5.8S rRNA)	5.8S	
SnF	U1A	125
SnG/ScG (5S rRNA)	5S I and II	
SnG'	5S III	120
SnH	4.5S I, II and III	96
SnI (tRNA)		
SnK		260
SnP		130
ScL (viral 7S)		260
ScM		180
ScD		180

Small Interfering RNA

Small interfering RNAs (siRNA) are the functional intermediates of **RNA interference** (RNAi, discussed later in this chapter), a defense in eukaryotic cells against viral invasion. In a process that is not yet completely understood, double-stranded RNA (dsRNA) species are believed to originate from transcription of inverted repeats or by the activity of cellular or viral RNA-directed RNA polymerases.[13] Biochemical analysis of RNA interference has revealed that these 21–22 nucleotide dsRNAs, also called **small intermediate RNAs**, are derived from successive cleavage of **dsRNAs** 500 or more nucleotide base pairs in length.[14] The ribonuclease III enzyme, dicer, is responsible for the generation of siRNA and another small RNA, micro RNA (see below), from dsRNA precursors.[15]

Transfer RNA

Translation of information from nucleic acid to protein requires reading of the mRNA by **ribosomes**, using adaptor molecules or **transfer RNA** (tRNA). Transfer RNAs are relatively short single-stranded polynucleotides of 73–93 bases in length, MW 24–31,000. There is at least one tRNA for each amino acid.

Advanced Concepts

The first demonstration of directed RNAi in the laboratory occurred in the experiments of Fire et al. with *C. elegans*.[45] These investigators injected dsRNA into worms and observed dramatic inhibition of the genes that generated the RNA. Since then, siRNAs have been introduced into plants and animals, including human cells growing in culture. Injection of long dsRNA kills human cells, but gene silencing can be achieved by introduction of the siRNAs or plasmids coding for the dsRNA. Libraries of siRNAs or DNA plasmids encoding them have been made that are complementary to over 8000 of the approximately 35,000 human genes.[54] These genetic tools have potential applications not only in identifying genes involved in disease but also as treatment for some of these diseases, particularly cancer where overexpression or abnormal expression of specific genes is part of the tumor phenotype.[55,56]

Advanced Concepts

Mitochondria contain distinct, somewhat smaller tRNAs.[57]

Advanced Concepts

The tRNA genes contain 14–20 extra nucleotides in the sequences coding for the anticodon loop that are transcribed into the tRNA. Enzymes that recognize other tRNA modifications remove these sequences (introns) by a cleavage-ligation process. Intron removal, addition of CCA to the 3′ end, and nucleotide modifications all occur following tRNA transcription. Enzymatic activities responsible for intron removal and addition of CCA may also contribute to intron removal and polyA addition to mRNA.

Eight or more of the nucleotide bases in all tRNAs are modified, usually methylated, after the tRNA synthesis. Most tRNAs have a guanylic residue at the 5′ end and the sequence C-C-A at the 3′ end. With maximum intrastrand hybridization, tRNAs take on a **cruciform structure** of double-stranded stems and single-stranded loops (Fig. 2-6). Transfer RNAs with longer sequences have an additional loop. The sequence C-C-A is found at the 3′ acceptor end of the tRNA. This is where the amino acid will be covalently attached to the tRNA. A seven-base loop (the **TψC loop**, where ψ stands for the modified nucleotide **pseudouridine**) contains the sequence 5′-TψCG-3′. The **variable loop** is larger in longer tRNAs. Another seven-base loop (the **anticodon loop**) contains the three-base pair **anticodon** that is complementary to the mRNA codon of its cognate amino acid. An 8-12–base loop (**D loop**) is relatively rich in **dihydrouridine**, another modified nucleotide. X-ray diffraction studies of pure crystalline tRNA reveal that the cruciform secondary structure of tRNA takes on an additional level of hydrogen bonding between the D loop and the TψC loop to form a γ-shaped structure (see Fig. 2-6).

Micro RNAs

Micro RNAs (**miRNA**) are tiny regulatory RNAs, 21-25 nt in length, derived from endogenous RNA **hairpin** structures (RNA folded into double-stranded states through intrastrand hydrogen bonds). Micro RNAs were discovered in the worm *Caenorhabditis elegans*. Two 22-nt RNAs were shown to contribute to temporal progres-

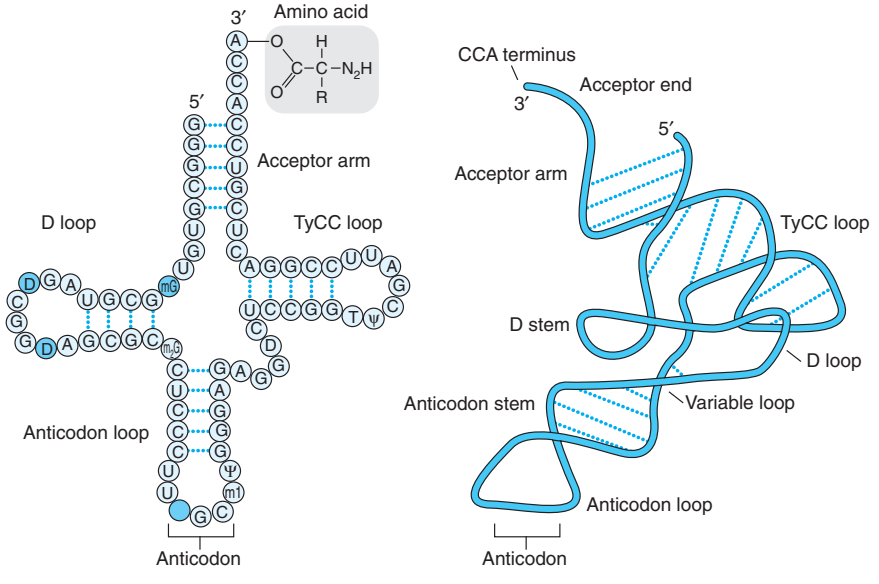


Figure 2-6 Alanine tRNA is an example of the general structure of tRNA, which is often depicted in a cruciform structure (left). The inverted "L" (right) is more accurate of the structure formed by intrastrand hydrogen bonding.

Historical Highlights

In 1964 Robert Holley and colleagues at Cornell University solved the first tRNA sequence. The sequence was that of alanine tRNA of yeast.[71] Yeast $tRNA^{ala}$ is 76 bases long; 10 of these bases are modified.

sion of cell fates by triggering down regulation of target mRNAs.[16-18] These RNA species were called **small temporal RNA** (stRNA).

Like siRNAs, these evolutionarily conserved RNAs are involved in control of gene expression. Unlike siRNAs that destroy mRNA, miRNAs pair with partially complementary sequences in mRNAs and inhibit translation. So far, over 100 miRNAs have been identified in eukaryotic cells and viruses.[19-22] Bacteria have genes that resemble miRNA precursors; however, the full miRNA system has not been demonstrated in bacteria. The true number of these RNAs may amount to thousands per genome.

Micro RNAs perform diverse functions in eukaryotic cells affecting gene expression, cell development, and defense. Because production of miRNAs is strictly regulated as to time or stage of cell development, finding them is a technical challenge. Many of these species are only present in virally infected cells or after introduction of foreign nucleic acid by transformation.[20] Novel approaches will be required for discovery of rare miRNAs expressed in specific cell types at specific times.

Other Small RNAs

Since the late 1990s a growing variety of small RNAs (sRNA) have been described in prokaryotes and eukaryotes, including **tiny noncoding RNAs** (tncRNA, 20-22 b),[23] **small modulatory RNA** (smRNA, 21-23b),[24] **small nucleolar RNAs** (snoRNA),[25] **tmRNA**[26] and others. In addition to RNA synthesis and processing, these molecules influence numerous cellular processes, including plasmid replication, bacteriophage development, chromosome structure, and development. These small untranslated RNA molecules have been termed sRNAs in bacteria and noncoding RNAs (ncRNAs) in eukaryotes.

Table 2.2 **RNA Polymerases**

Enzyme	Template	Product
E. coli RNA polymerase II	DNA	mRNA
RNA polymerase I	DNA	rRNA
RNA polymerase II	DNA	mRNA
RNA polymerase III	DNA	tRNA, snRNA
Mitochondrial RNA polymerase	DNA	mRNA
Mammalian DNA polymerase α	DNA	primers
HCV RNA polymerase	RNA	Viral genome
Dengue virus RNA polymerase	RNA	Viral genome
PolyA polymerase	None	PolyA tails

RNA Polymerases

RNA synthesis is catalyzed by **RNA polymerase** enzymes (Table 2.2). One multi-subunit prokaryotic enzyme is responsible for the synthesis of all types of RNA in the prokaryotic cell. Eukaryotes have three different RNA polymerase enzymes. DNA-dependent RNA polymerases require a DNA template. RNA-dependent RNA polymerases require an RNA template.

In prokaryotes, all types of RNA are synthesized by a single RNA polymerase. Bacterial RNA polymerase consists of five subunits, two α and one of each β, β' and σ[27] (Fig. 2-7).

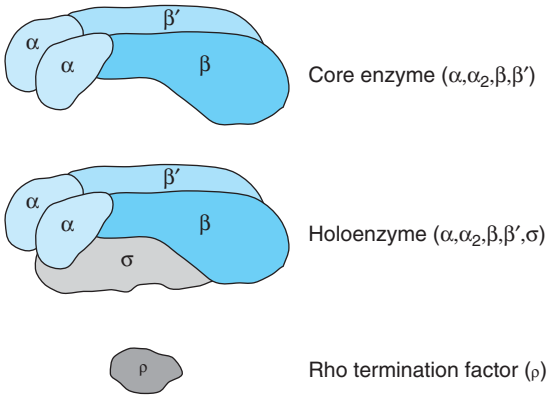


Figure 2-7 Prokaryotic RNA polymerase is made up of separate proteins. The four subunits that make up the core enzyme have the capacity to synthesize RNA. The sigma cofactor aids in accurate initiation of RNA synthesis. The rho cofactor aids in termination of RNA synthesis.

Table 2.3 **Cellular Location and Activity of RNA pol I, II, and III in Eukaryotes**

Type	Location	Products	α-Amanitin
I	Nucleolus	18s, 5.8s, 28s rRNA	Insensitive
II	Nucleus	mRNA, snRNA	Inhibited
III	Nucleus	tRNA, 5s rRNA	Inhibited by high concentration

Burgess et al.[28] showed that the α2,β,β' **core enzyme** retained the catalytic activity of the α2,β,β'σ complete, or **holoenzyme**, suggesting that the **sigma factor** played no role in RNA elongation. In fact, the sigma factor is released at RNA initiation. The role of sigma factor is to guide the complete enzyme to the proper site of initiation on the DNA.

In eukaryotes, there are three multisubunit nuclear DNA-dependent RNA polymerases, RNA polymerase I, II, and III (Table 2.3). A single subunit mitochondrial RNA polymerase, imported to organelles, is also encoded in the nucleus. The three RNA polymerases in eukaryotic cells were first distinguished by their locations in the cell. **RNA polymerase I** (pol I) is found in the nucleolus. **RNA polymerase II** (pol II) is found in the nucleus. **RNA polymerase III** (pol III), one of the first nucleic acid polymerases discovered, is also found in the nucleus (and sometimes the cytoplasm).

Advanced Concepts

The three polymerases were also distinguished by their differential sensitivity to the toxin **α-amanitin**.[58-60] This toxin is a bicyclic octapeptide isolated from the poisonous mushroom *Amanita phalloides*. Pol II is sensitive to relatively low amounts of this toxin. Pol III is sensitive to intermediate levels, and pol I is resistant. This toxin has been invaluable in the research setting to determine which polymerase activity is responsible for synthesis of newly discovered types of RNA and to dissect the biochemical properties of the polymerases.[61-63]

Advanced Concepts

The most well-studied eukaryotic RNA polymerase II is from the yeast *Saccharomyces cervisiae*. It is a 0.4 megadalton complex of 12 subunits. The yeast enzyme works in conjunction with a large complex of proteins required for promoter recognition and melting, transcription initiation, elongation and termination, and transcript processing (splicing, capping, and polyadenylation).

The differential drug sensitivity, cellular location, and ionic requirements of the three eukaryotic RNA polymerases were used to assign the polymerases to the type of RNA they synthesize. Pol I synthesizes rRNA (except 5S rRNA). Pol II synthesizes mRNAs and snRNAs. Pol III synthesizes tRNAs, 5S rRNA, and some snRNAs. Pol II (also called RNA polymerase b [Rpb]) is the central transcribing enzyme of mRNA in eukaryotes.

RNA viruses carry their own **RNA-dependent RNA polymerases**. Hepatitis C virus and Dengue virus carry this type of polymerase to replicate their RNA genomes. RNA-dependent RNA polymerase activity has also been found in lower eukaryotes.[29] The purpose of these enzymes in cells may be associated with RNAi and gene silencing.

PolyA polymerase is a template-independent RNA polymerase. This enzyme adds adenine nucleotides to the 3′ end of mRNA.[30] The resulting **polyA tail** is important for mRNA stability and translation into protein (see below).

Other RNA-Metabolizing Enzymes

Ribonucleases

Ribonucleases degrade RNA in a manner similar to the degradation of DNA by deoxyribonucleases (Table 2.4). An endoribonuclease, cleavage and polyadenylation specific factor (CPSF) is required for proper termination of RNA synthesis.[32,33] Along with RNA polymerase II subunits and other proteins, this enzyme cuts the nascent RNA transcript before addition of the polyA tail by polyA polymerase.

Advanced Concepts

With advances in crystallography, the molecular mechanisms of RNA synthesis are being revealed. During transcription, DNA enters a positively charged cleft between the two largest subunits of the RNA polymerase. At the floor of the cleft is the active site of the enzyme to which nucleotides are funneled through a pore in the cleft beneath the active site (pore 1). In the active site, the DNA strands are separated, and the RNA chain is elongated driven by cleavage of phosphates from each incoming ribonucleoside triphosphate. The resulting DNA-RNA hybrid moves out of the active site nearly perpendicular to the DNA coming into the cleft. After reaching a length of 10 bases, the newly synthesized RNA dissociates from the hybrid and leaves the complex through an exit channel. Three protein loops, "rudder," "lid," and "zipper," are involved in hybrid dissolution and exit of the RNA product.[64,65]

RNA Helicases

RNA helicases catalyze the unwinding of double-stranded RNA. RNA synthesis and processing require the activity of helicases. These enzymes have been characterized in prokaryotic and eukaryotic organisms. Some RNA helicases work exclusively on RNA. Others can work on DNA:RNA heteroduplexes and DNA substrates.

Advanced Concepts

There are two major groups of RNA helicases defined by the amino acid sequence of their conserved regions. **DEAD-box helicases** (asp-glu-ala-asp) are typified by the yeast translation initiation factor e1F4A, which unwinds messenger RNA at the 5′ untranslated end for proper binding of the small ribosomal subunit. DEAD-box proteins are not strong helicases and may be referred to as **unwindases** or **RNA chaparones**.[66] The DEAH-box helicases (asp-glu-ala-his) (**DExDH-box helicases**) resemble DNA helicases. They act on mRNA and snRNA during splicing. They may also be required for ribosome biosynthesis. In addition to RNA unwinding, protein dissociation, splicing, and translation, RNA helicases also participate in RNA turnover and chromatin remodeling.

Another activity of these enzymes is in the removal of proteins from RNA-protein complexes.

Regulation of Transcription

Gene expression is a key determinant of phenotype. The sequences and factors controlling when and how much protein is synthesized are equally as important as the DNA sequences encoding the amino acid makeup of a protein. Early studies aimed at the characterization of

Table 2.4 **RNases Used in Laboratory Procedures[49]**

Enzyme	Source	Type	Substrate
RNAse A	Bovine	Endoribonuclease	Single-stranded RNA 3′ to pyrimidine residues
RNAse T1	*Aspergillus*	Endoribonuclease	3′ Phosphate groups of guanines
RNAse H	*E. coli*	Exoribonuclease	RNA hybridized to DNA
RNAse CL3	*Gallus*	Endoribonuclease	RNA next to cytidylic acid
Cereus RNAse	*Physarum*	Endoribonuclease	Cytosine and uracil residues in RNA
RNAse Phy M	*Physarum*	Endoribonuclease	Uracil, adenine, and guanine residues in RNA
RNAse U2	*Ustilago*	Endoribonuclease	3′ Phosphodiester bonds next to purines
RNAse T2	*Aspergillus*	Endoribonuclease	All phosphodiester bonds, preferably next to adenines
S1 nuclease	*Aspergillus*	Exoribonuclease	RNA or single-stranded DNA
Mung bean nuclease	Mung bean sprouts	Exoribonuclease	RNA or single-stranded DNA
RNAse Phy I	*Physarum*	Exoribonuclease	Guanine, adenine, or uracil residues in RNA

gene structure were confounded by phenotypes that resulted from aberrations in gene expression rather than in protein structural alterations.

Gene expression is tightly regulated throughout the life of a cell. Because gene products often function together to bring about a specific cellular response, specific combinations of proteins in stoichiometric balance are crucial for cell differentiation and development. Protein availability and function are controlled at the levels of transcription, translation, and protein modification and stability. The most immediate and well-studied level of control of gene expression is transcription **initiation**. Molecular technology has led to an extensive study of transcription initiation, so a large amount of information on gene expression refers to this level of transcription.

Two types of factors are responsible for regulation of RNA synthesis: **cis factors** and **trans factors** (Fig. 2-8). cis factors are DNA sequences that mark places on the DNA involved in the initiation and control of RNA synthesis. Trans factors are proteins that bind to the cis sequences and direct the assembly of transcription complexes at the proper gene. In order for transcription to occur, several proteins must assemble at the gene's transcription initiation site including specific and general transcription factors and the RNA polymerase complex.

An **operon** is a series of structural genes transcribed together on one mRNA and subsequently separated into individual proteins. In organisms with small genomes

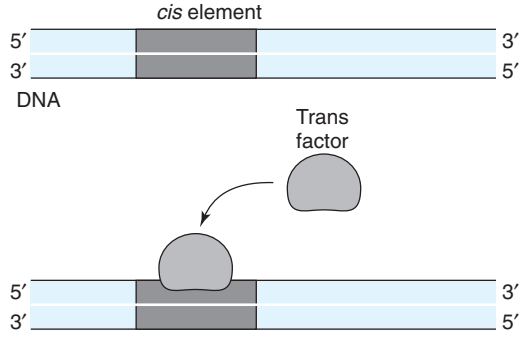


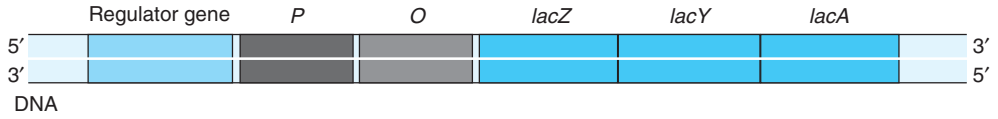
Figure 2-8 cis factors or cis elements (top) are DNA sequences recognized by transcription factors, or trans factors, or DNA binding proteins (bottom).

such as bacteria and viruses, operons bring about coordinate expression of proteins required at the same time; for example, the enzymes of a metabolic pathway. The lactose operon contains three structural genes, *LacZ, LacY,* and *LacA*, which are all required for the metabolism of lactose. The *LacZ* gene product, β-galactosidase, hydrolyzes lactose into glucose and galactose. The *LacY* gene product, lactose permease, transports lactose into the cell. The *LacA* gene product, thiogalactoside transacetylase, transacetylates galactosides. The *LacI* gene, which encodes a protein **repressor** and the repressor's binding site in the DNA just 5′ to the start of the operon, is responsible for the regulated expression of the operon. When *E. coli* is growing on glucose as a carbon source, the lactose-metabolizing enzymes are not required, and this operon is minimally expressed. Within 2–3 minutes after shifting the organism to a lactose-containing medium, the expression of these enzymes is increased a thousandfold.

Fig. 2-9 shows a map of the lac operon. The three structural genes of the operon are preceded by the promoter, where RNA polymerase binds to the DNA to start transcription and the cis regulatory element, the **operator**, where the regulatory repressor protein binds. The sequences coding for the repressor protein are located just 5′ to the operon. In the absence of lactose, the repressor protein binds to the operator sequence and prevents transcription of the operon. When lactose is present, it binds to the repressor protein and changes its conformation and lowers its affinity to bind the operator sequence. This results in expression of the operon (Fig. 2-10). Jacob and Monod deduced these details through analysis of a series of mutants. Since their work, numerous regulatory

Historical Highlights

The production of particular proteins in media containing specific substrates was observed early in the last century, a phenomenon termed **enzyme adaptation**. It was later called **induction**, and detailed analysis of the lactose operon was the first description of an inducible gene expression at the molecular level. The effect of gene expression on phenotype was initially demonstrated by Monod and Cozen-Bazire in 1953 when they showed that synthesis of tryptophane in *Aerobacter* was inhibited by tryptophane.[72] Jacob and Monod subsequently introduced the concept of two types of genes, structural and regulatory in an inducible system, the lactose operon in *E. coli*.[73]



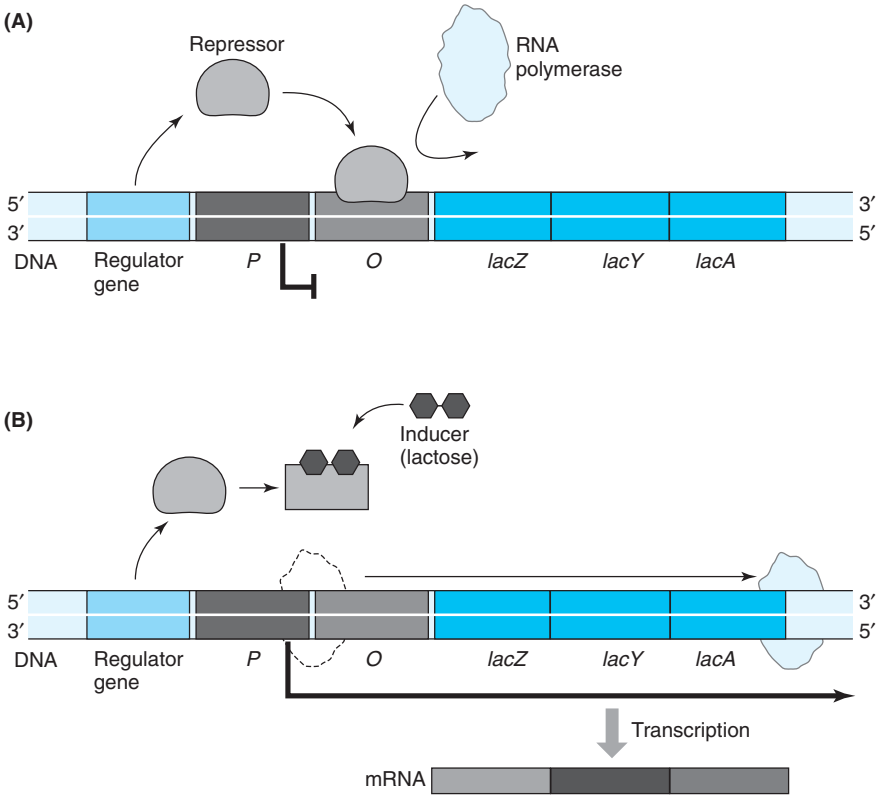
■ Figure 2-9 General structure of the lac operon. The regulator or repressor gene codes for the repressor protein trans factor that binds to the operator.

systems have been described in prokaryotes and eukaryotes, all using the same basic idea of combinations of cis and trans factors.

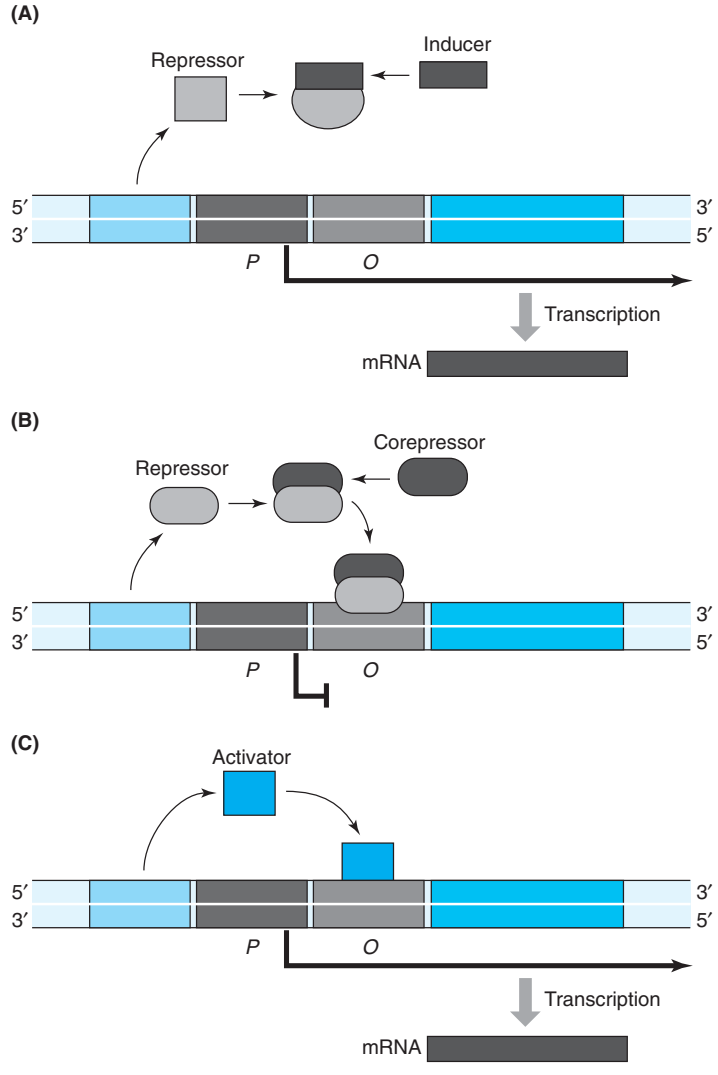
Other operons are controlled in a similar manner by the binding of regulatory trans factors to cis sequences preceding the structural genes (Fig. 2-11). A different type of negative control is that found in the arg operon where a **corepressor** must bind to a repressor in order to turn off transcription (enzyme repression). Compare this with the inducer that prevents the repressor from binding the operator to turn on expression of the lac operon (enzyme induction). The mal operon is an example of positive control where an **activator** binds with RNA polymerase to turn on transcription.

Another mechanism of control in bacteria is **attenuation**. This type of regulation works through formation of stems and loops in the RNA transcript by intrastrand hydrogen bonding. These secondary structures allow or prevent transcription, for instance by exposing or sequestering ribosome binding sites at the beginning of the transcript.

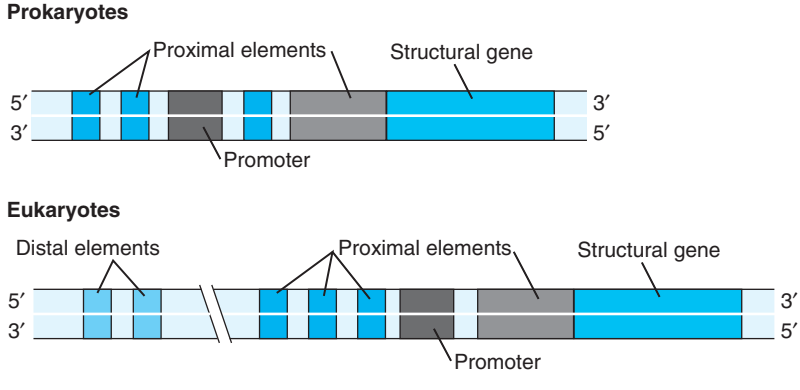
The general arrangement of cis factors on DNA is shown in Fig. 2-12. These sequences are usually 4–20 bp in length. Some are inverted repeats with the capacity to form a cruciform structure in the DNA duplex recognizable by specific proteins. Prokaryotic regulatory sequences are found within close proximity of the gene. Eukaryotic genes have both **proximal** and **distal** regula-



■ Figure 2-10 Two states of the lac operon. (A) The repressor protein (R) binds to the operator cis element (O) preventing transcription of the operon from the promoter (P). (B) In the presence of the inducer lactose, the inducer binds to the repressor, changing its conformation, decreasing its affinity for the operator, allowing transcription to occur.



■ Figure 2-11 Modes of regulation in prokaryotes include induction as found in the lac operon (A), repression as found in the arg operon (B), and activation as in the mal operon (C).



■ Figure 2-12 cis regulatory elements in prokaryotes are located close to the structural genes they control in the vicinity of the promoter. In eukaryotes, distal elements can be located thousands of base pairs away from the genes they control. Proximal elements can be located in or around the genes they control. Elements may also be located behind their target genes.

Advanced Concepts

Unlike prokaryotes, eukaryotes do not have operons. Coordinately expressed genes can be scattered in several locations. Synchronous expression is brought about in eukaryotes by **combinatorial control**. Genes that are expressed in a similar pattern share similar cis elements so that they respond simultaneously to specific combinations of controlling trans factors.

tory elements. Distal eukaryotic elements can be located thousands of base pairs away from the genes they control. **Enhancers** and **silencers** are examples of distal regulatory elements that respectively stimulate or dampen expression of distant genes.

Epigenetics

Histone Modification

Gene activity (transcription) can be altered in ways other than by cis elements and transcription factors. Histone modification, DNA methylation, and gene silencing by double-stranded or antisense RNA regulate gene expression. These types of gene regulation are called **epigenetic regulation**.

Chromatin is nuclear DNA that is compacted onto nucleosomes. A **nucleosome** is about 150 bases of DNA wrapped around a complex of eight **histone** proteins, two each of H2A, H2B, H3, and H4. Histones are not only structural proteins but can also regulate access of trans factors and RNA polymerase to the DNA helix. Modification of histone proteins affects the activity of chromatin-associated proteins and transcription factors that increase or decrease gene expression. Through these protein interactions, chromatin can move between transcriptionally active and transcriptionally silent states. Different types of DNA modifications, including methylation, phosphorylation, ubiquitination, and acetylation, establish a complex system of control. These modifications create a docking surface that forms an environment for interaction with other chromatin binding factors and, ultimately, regulatory proteins (Fig. 2-13).[34] A collection of such architectural and regulatory proteins assembled on an enhancer element has been termed an **enhanceosome.**[35]

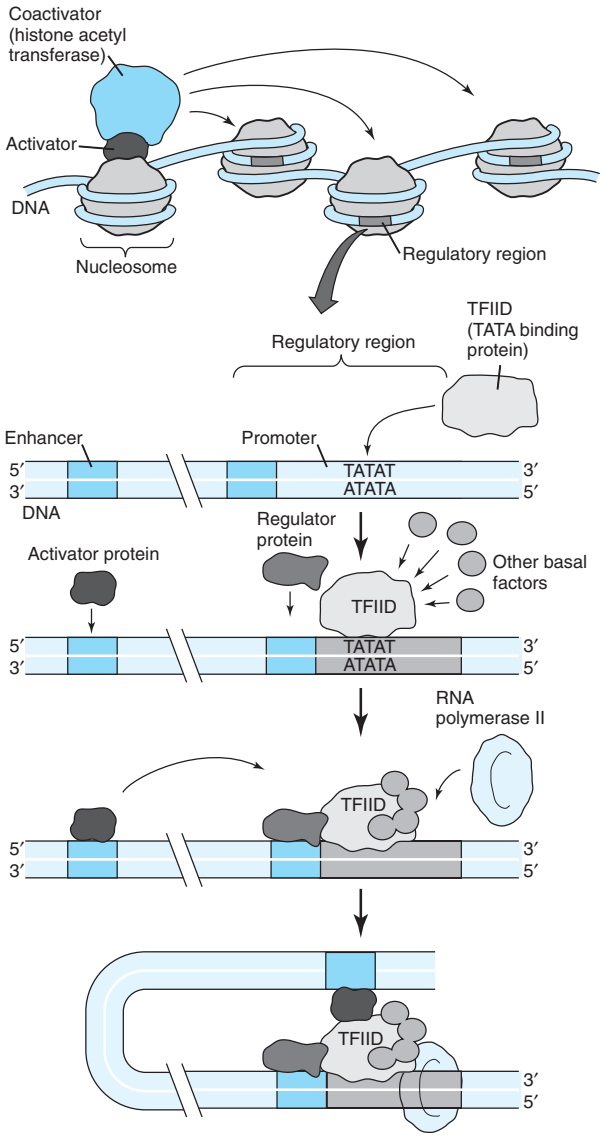
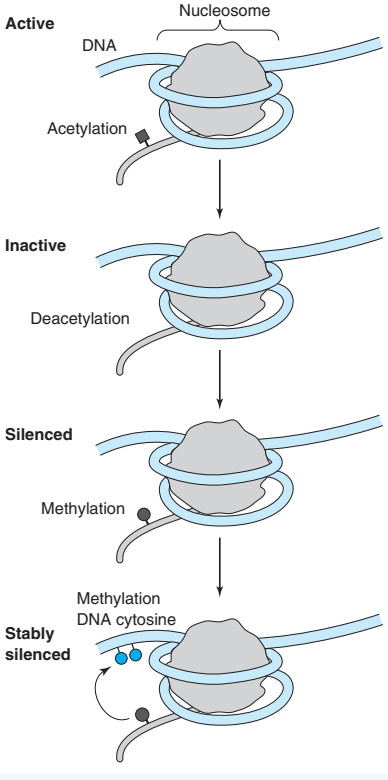


Figure 2-13 Interaction of transcription factors and nucleosome acetylation at the regulatory region of a gene (top) induce assembly of protein factors and RNA polymerase at the promoter (bottom).

The involvement of histone modification with gene expression has led to the study of aberrations in these modifications in disease states such as viral infections and neoplastic cells. Thus, analysis of histone states may be another target for diagnostic, prognostic, and therapeutic applications.

Advanced Concepts

One theory holds that pre-existing and gene-specific histone modifications constitute a histone code that extends the information potential of the genetic code.[67] Accordingly, **euchromatin**, which is transcriptionally active, has more acetylated histones and less methylated histones than transcriptionally silent **heterochromatin** made up of more condensed nucleosome fibers. Although methylated histones can activate transcription by recruiting histone acetylases, establishment of localized areas of histone methylation can also prevent transcription by recruiting proteins for heterochromatin formation and is one form of gene or transcriptional silencing.[68] Transcriptional silencing is responsible for inactivation of the human X chromosome in female embryo development and **position effects**, the silencing of genes when placed in heterochromatic areas.



Deacetylation and methylation of histones can establish silenced regions of DNA.

DNA Modification

DNA methylation is another type of epigenetic regulation of gene expression in eukaryotes and prokaryotes. In vertebrates, methylation occurs in cytosine-guanine—rich sequences in the DNA (**CpG islands**) (Fig. 2-14). CpG islands were initially defined as regions >200 bp in length with an observed/expected ratio of the occurrence of CpG >0.6.[36] This definition may be modified to a more selective GC content to exclude unrelated regions of naturally high GC content.[37] CpG islands are found around the first exons, promoter regions, and sometimes toward the 3′ ends of genes. Aberrant DNA methylation at these sites is a source of dysregulation of genes in disease states. Methylation of cytosine residues in the promoter regions of tumor suppressor genes is a mechanism of inactivation of these genes in cancer.[38] Methods to analyze promoter methylation have been developed.[39,40]

Methylation of DNA is the main mechanism of genomic **imprinting**, the gamete-specific silencing of genes.[41,42] Imprinting maintains the balanced expression of genes in growth and embryonic development by selective methylation of homologous genes. This controlled methylation occurs during gametogenesis and is different in male and female gametes. A convenient illustration of imprinting is the comparison of mules and hinnies. A mule (progeny of a female horse and male donkey) has a distinct phenotype from that of a hinny (progeny of a male horse and a female donkey). The difference is due to distinct imprinting of genes inherited through the egg versus those inherited through the sperm. Genetic diseases in humans, Angelman's syndrome and Prader-Willi syndrome, are clinically distinct conditions that result from the same genetic defect on chromosome 15. The phenotypic differences depend on whether the genetic lesion involves the maternally or paternally inherited chromosome. Imprinting may be partly responsible for abnormal development and phenotypic characteristics of

...GGAGGAGCGCGCGGCGGCGGCCAGAGA

AAAGCCGCAGCGGCGCGCGCGCACCCGGA

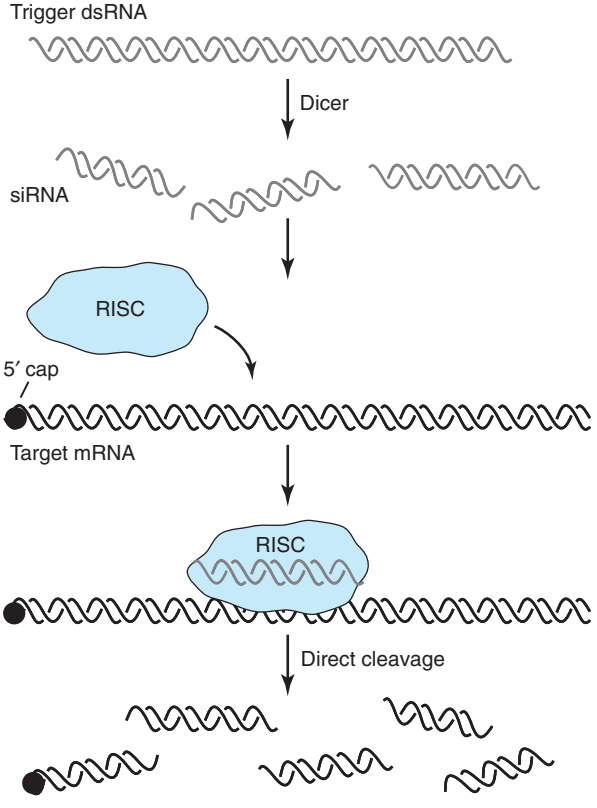
CAGCCGGCGGAGGCGGG...

Figure 2-14 CpG islands are sequences of DNA rich in the C-G dinucleotides. These structures have no specific sequence other than a higher than expected occurrence of CpG.

cloned animals as the process of cloning by nuclear transfer bypasses gametogenesis.[43,44]

RNAi

Another phenomenon that affects transcription is **RNAi.** First discovered in the worm *Caenorhabditis elegans* in 1993,[16,18,45] RNAi is mediated siRNAs.[46] The siRNAs and other proteins assemble into an enzyme complex, called the RNA induced silencing complex (**RISC**) (Fig. 2-15). The RISC uses the associated siRNA to bind and degrade mRNA with sequences complementary to the siRNA. In this way translation of specific genes can be inhibited. siRNAs may also guide methylases to homologous sequences on the chromosome, either through direct interaction with the methylating enzymes or by hybridizing to specific sequences in the target genes, forming a bulge in the DNA molecule that attracts methylases.



■ Figure 2-15 Trigger double-stranded RNA is cleaved into siRNAs that become part of the RISC. Led by the complementary siRNA sequences, RISC binds to the target RNA and begins RNA cleavage.[13]

Advanced Concepts

RNA interference has been utilized as a method to specifically inactivate genes in the research laboratory. Preferable to gene deletions or "knock-out" methods that take months to inactivate a single gene, RNAi can specifically inactivate several genes in a few days.

Initially, this method did not work well in mammalian cells, due to a cellular response elicited by introduction of long dsRNAs that turns off multiple genes and promotes cell suicide. Studies with shorter dsRNAs, however, have been demonstrated to work in mammalian cell cultures [31]. This type of specific gene control in vitro may lead to control cell cultures or transcriptional states useful in the clinical laboratory setting as well as the research laboratory.

RNAi, however is not always required for DNA methylation.[47] Alternatively, siRNAs may bind to specific sequences in already transcribed homologous RNA, targeting them for degradation into more siRNAs.

RNAi can also control gene expression during development. There are more than 200 regulatory RNAi's in humans. Mice without RNAi function do not survive long into gestation, emphasizing a key role of this mechanism in embryogenesis.

Because of the high specificity of siRNAs, RNAi has also been proposed as a manner of gene and viral therapy.[48] Silencing has been targeted to growth-activating genes such as the vascular endothelial growth factor in tumor cells. Small interfering RNA silencing may also be aimed at HIV and influenza viruses. As with other gene therapy methods, stability and delivery of the therapeutic siRNAs are major challenges.

• STUDY QUESTIONS •

RNA Secondary Structure

1. Draw the secondary structure of the following RNA. The complementary sequences (inverted repeat) are underlined.
   5′CAUGUUCAGCUCAUGUGAACGCU 3′

2. Underline the rest of the *two* inverted repeats in the following RNA, then draw the secondary structure.
   5′CUGAACUUCAGUCAAGCAUGCACU-GAUGCUU 3′

The Lac Operon

1. Using the depiction of the lac operon in Figures 2-9 and 2-10, indicate whether gene expression (transcription) would be on or off under the following conditions:
   (P = promoter; O = operator; R = repressor)
   a. P+ O+ R+, no inducer present - OFF
   b. P+ O+ R+, inducer present - ON
   c. P- O+ R+, no inducer present -
   d. P- O+ R+, inducer present -
   e. P+ O- R+, no inducer present -
   f. P+ O- R+, inducer present -
   g. P+ O+ R-, no inducer present -
   h. P+ O+ R-, inducer present -
   i. P- O- R+, no inducer present -
   j. P- O- R+, inducer present -
   k. P- O+ R-, no inducer present -
   l. P- O+ R-, inducer present -
   m. P+ O- R-, no inducer present -
   n. P+ O- R-, inducer present -
   o. P- O- R-, no inducer present -
   p. P- O- R-, inducer present -

Epigenetics

1. Indicate whether the following events would increase or decrease expression of a gene:
   a. Methylation of cytosine bases 5′ to the gene
   b. Histone acetylation close to the gene
   c. siRNAs complementary to the gene transcript

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Chapter 3 *Lela Buckingham*

Proteins

OUTLINE

OBJECTIVES

- Describe the structure and chemical nature of the 20 amino acids.
- Show how the chemistry of the amino acids affects the chemical characteristics and functions of proteins.
- Define primary, secondary, tertiary, and quaternary structure of protein organization.
- Give the definition of a gene.
- Recount how the genetic code was solved.
- Describe how amino acids are polymerized into proteins, using RNA as a guide (translation).

Proteins are the products of transcription and translation of the nucleic acids. Even though nucleic acids are most often the focus of molecular analysis, the ultimate effect of the information stored and delivered by the nucleic acid is manifested in proteins. Analysis of the amount and mutational status of specific proteins has long been performed in situ using immunohistochemistry, on live cells using flow cytometry, and on isolated proteins by Western blots. More recently, global protein analysis by mass spectrometry (proteomics) has been applied to clinical work on a research basis. Even if proteins are not being tested directly, they manifest the phenotype directed by the nucleic acid information. In order to interpret results of nucleic acid analysis accurately, therefore, it is important to understand the movement of genetic information from DNA to protein as dictated by the genetic code.

Amino Acids

Proteins are polymers of **amino acids**. Each amino acid has characteristic biochemical properties determined by the nature of its amino acid **side chain** (Fig. 3-1). Amino acids are grouped according to their polarity (tendency to interact with water at pH 7) as follows: nonpolar, uncharged polar, negatively-charged polar, and positively-charged polar (Table 3.1).

The properties of amino acids that make up a protein determine the shape and biochemical nature of the protein, such as highly charged, hydrophilic, or hydrophobic. A single protein can have separate domains with different properties. For example, transmembrane proteins might have several stretches of hydrophobic amino acids that will sit in the lipid membrane of the cell; they might also have hydrophilic or charged extracellular domains (Fig. 3-2).

Amino acids are synthesized in vivo by stereospecific enzymes so that naturally occurring proteins are made of amino acids of L-stereochemistry. The central asymmetric carbon atom is attached to a carboxyl group, an amino group, a hydrogen atom, and the side chain. Proline differs somewhat from the rest of the amino acids in that its side chain is cyclic, the amino group attached to the end carbon of the side chain making a five-carbon ring (see Fig. 3-1).

Amino acids are also classified by their biosynthetic origins or similar structures based on a common biosynthetic precursor (Table 3.2). Histidine has a unique synthetic pathway using metabolites common with purine nucleotide biosynthesis, which affords connection of amino acid synthesis with nucleotide synthesis.

Table 3.1 **Classification of Amino Acids Based on Polarity of Their Side Chains**

Classification	Amino Acid	Abbreviations
Nonpolar	Alanine	Ala, A
	Isoleucine	Ile, I
	Leucine	Leu, L
	Methionine	Met, M
	Phenylalanine	Phe, F
	Tryptophan	Trp, W
	Valine	Val, V
Polar	Asparagine	Asn, N
	Cysteine	Cys, C
	Glutamine	Gln, Q
	Glycine	Gly, G
	Proline	Pro, P
	Serine	Ser, S
	Threonine	Thr, T
	Tyrosine	Tyr, Y
Negatively charged (acidic)	Aspartic acid	Asp, D
	Glutamic acid	Glu, E
Positively charged (basic)	Arginine	Arg. R
	Histidine	His, H
	Lysine	Lys, K

At pH 7, most of the carboxyl groups of the amino acids are ionized, and the amino groups are not. The ionization can switch between the amino and carboxyl groups, making the amino acids **zwitterions** at physiological pH (Fig. 3-3). At a specific pH, amino acids will become completely positively or negatively charged. These are the **pK** values. At the pH where an amino acid is neutral, its positive and negative charges are in balance. This is the **pI** value. Each amino acid will have its characteristic pI. The pI of a peptide or protein is determined by the ionization state (positive and negative charges) of the side chains of its constituent amino acids.

The amino and carboxyl terminal groups of the amino acids are joined in a carbon-carbon-nitrogen (-C-C-N-) substituted amide linkage (**peptide bond**) to form the protein backbone (Fig. 3-4). Two amino acids joined together by a peptide bond make a **dipeptide**. Peptides

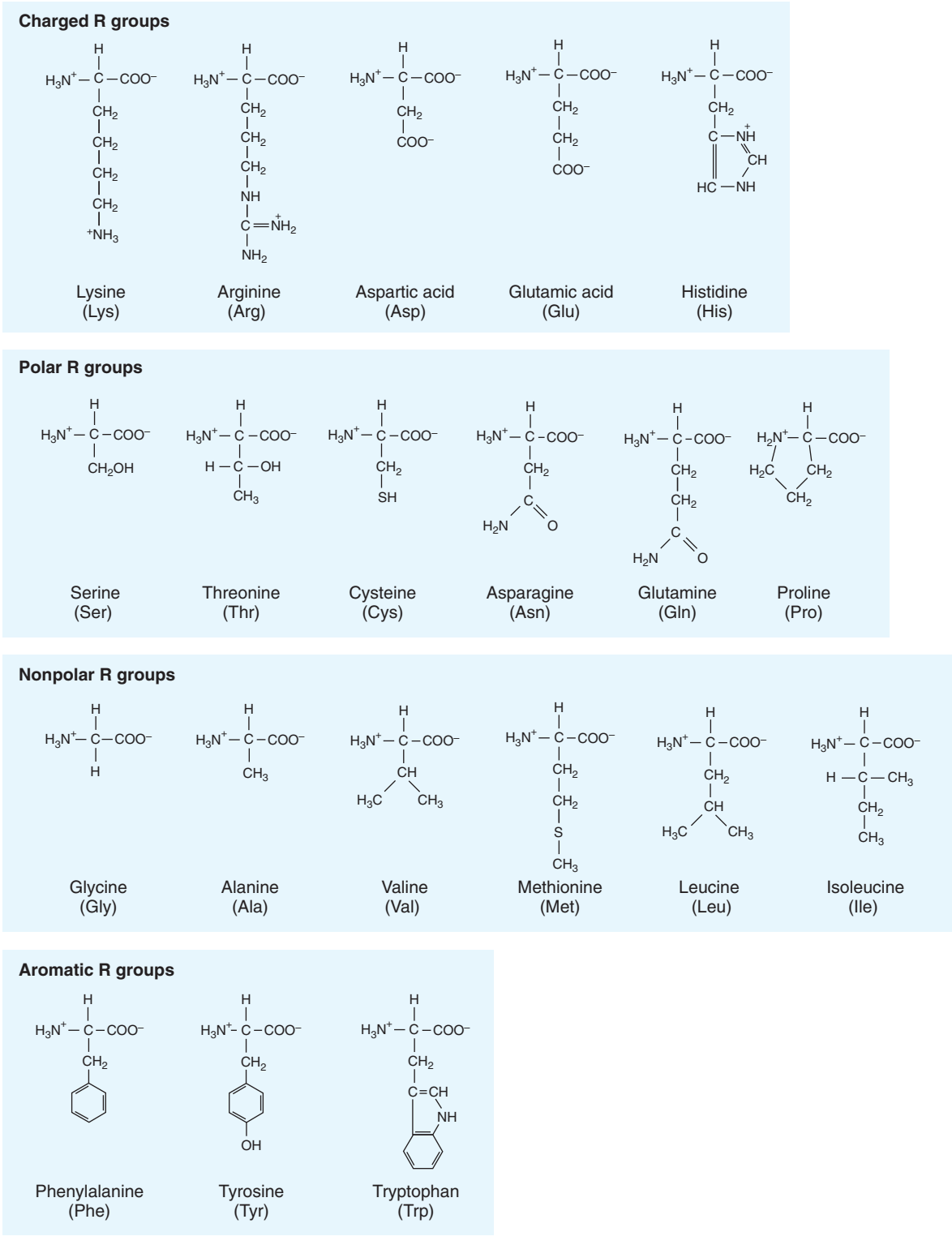


Figure 3-1 Structures of the 20 amino acids. The side chains are grouped according to their chemical characteristics.

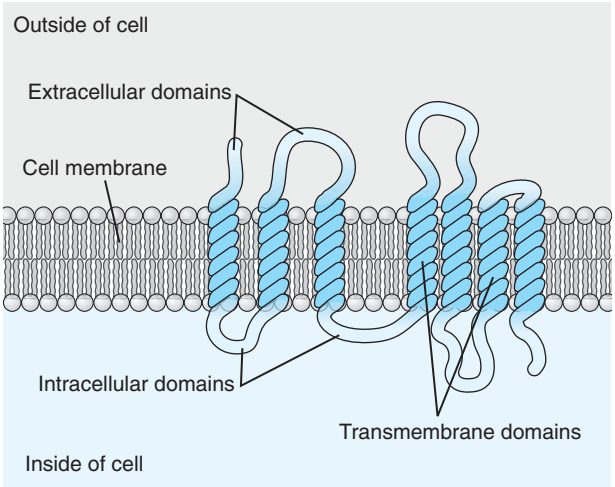


Figure 3-2 Transmembrane proteins have hydrophobic transmembrane domains and hydrophilic domains exposed to the intracellular and extracellular spaces. The biochemical nature of these domains results from their distinct amino acid compositions.

with additional units are tri-, tetra-, pentapeptides, and so forth, depending on how many units are attached to each other. At one end of the peptide will be an amino group (the **amino terminal** or NH_2 end), and at the opposite terminus of the peptide will be a carboxyl group (the **carboxy terminal** or COOH end). Like the 5′ to 3′ direction of nucleic acids, peptide chains grow from the amino to the carboxy terminus.

Proteins are **polypeptides** that can reach sizes of more than a thousand amino acids in length. Proteins constitute the most abundant macromolecules in cells. The information stored in the sequence of nucleotides in DNA is transcribed and translated into an amino acid sequence that will ultimately bring about the genetically coded phenotype.

The collection of proteins produced by a genome is a **proteome**. The proteome of humans is larger than the genome, possibly 10 times its size.[1] This is because a single gene can give rise to more than one protein through alternate splicing and other post-transcriptional/post-translational modifications.

The sequence of amino acids in a protein determines the nature and activity of that protein. This sequence is the **primary structure** of the protein and is read by convention from the amino terminal end to the carboxy terminal end. Minor changes in the primary structure can alter the activity of proteins dramatically. The single amino acid substitution that produces hemoglobin S in

Advanced Concepts

An unusual 21st amino acid, selenocysteine, is a component of **selenoproteins** such as glutathione peroxidase and formate dehydrogenase. There are 25 known mammalian selenoproteins. Selenocysteine is cysteine with the sulfur atom replaced by selenium.[31] Selenocysteine is coded by a predefined UGA codon that inserts selenocysteine instead of a termination signal.

Table 3.2 **Amino Acid Biosynthetic Groups**

Biosynthetic Group	Precursor	Amino Acids
α-Ketoglutarate group	α-Ketoglutarate	Gln, Q
		Glu, E
		Pro, P
		Arg. R
Pyruvate group	Pyruvate	Ala, A
		Val, V
		Leu, L
Oxalate group	Oxalacetic acid	Asp, D
		Asn, N
		Lys, Q
		Ile, I
		Thr, T
		Met, M
Serine group	3-Phosphoglycerate	Gly, G
		Ser, S
		Cys, C
Aromatic group	Chorismate	Phe, F
		Trp, W
		Tyr, Y
(Unique biosynthesis)		His, H

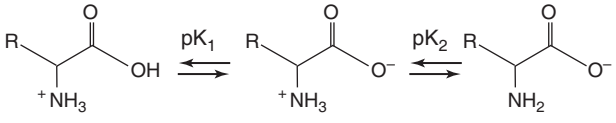


Figure 3-3 An amino acid is positively charged at pK_1 and negatively charged at pK_2. At the pH where the positive and negative charges balance (pI), the molecule is neutral.

Advanced Concepts

Peptides can have biological activity. Hormones such as insulin, glucagons, corticotropin, oxytocin, bradykinin, and thyrotropin are examples of peptides (9-40 amino acids long) with strong biological activity. Several antibiotics such as penicillin and streptomycin are also peptides.

sickle cell anemia is a well-known example. Replacement of a soluble glutamine residue with a hydrophobic valine at the sixth residue changes the nature of the protein so that it packs aberrantly in corpuscles and drastically alters cell shape. Minor changes in primary structure can have such drastic effects because the amino acids must often cooperate with one another to bring about protein structure and function.

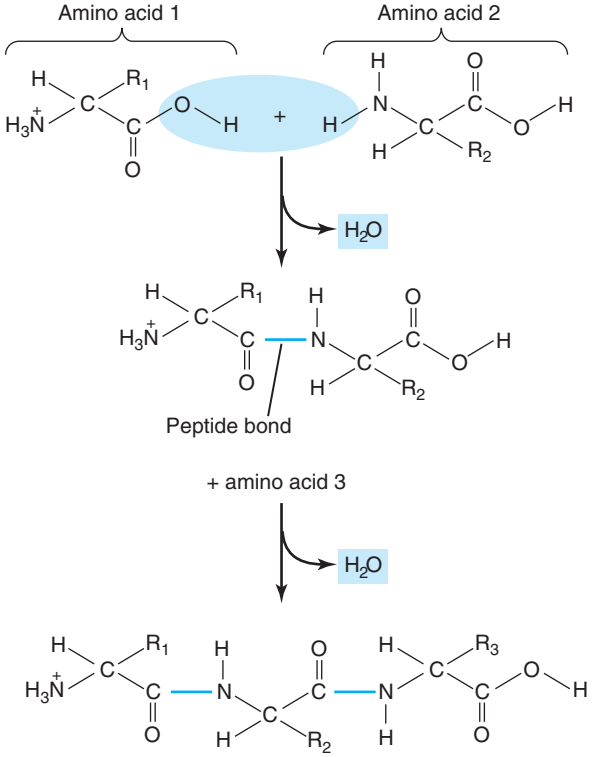


Figure 3-4 The peptide bond is a covalent linkage of the carboxyl C of one amino acid with the amino N of the next amino acid. One molecule of water is released in the reaction.

Historical Highlights

The primary sequence of proteins can be determined by a method first described in the report of the amino acid sequence of insulin by Fred Sanger.[38,39] This procedure was carried out in six steps. First, the protein was dissociated to amino acids. The dissociation products were separated by ion exchange chromatography in order to determine the type and amount of each amino acid. Second, the amino terminal and carboxy terminal amino acids were determined by labeling with 1-fluoro-2,4-dinitrobenzene and digestion with carboxypeptidase, respectively. The complete protein was then fragmented selectively at lysines and arginines with trypsin to 10–15 amino acid peptides. Fourth, Edmund degradation with phenylisothiocyanate and dilute acid labeled and removed the amino terminal residue. This was repeated on the same peptide until all the amino acids were identified. Fifth, after another selected cleavage of the original protein using cyanogen bromide, chymotrypsin, or pepsin and identification of the peptides by chromatography, the peptides were again sequenced using the Edmund degradation. Sixth, the complete amino acid sequence could be assembled by identification of overlapping regions.

Interactions between amino acid side chains fold a protein into predictable configurations. These include ordered beta or **beta-pleated sheets** and less ordered **alpha helices** or **random coils**. The alpha helix and beta sheet structures in proteins (Fig. 3-5) were first described by Linus Pauling and Robert Corey in 1951.[2-5] This level

Advanced Concepts

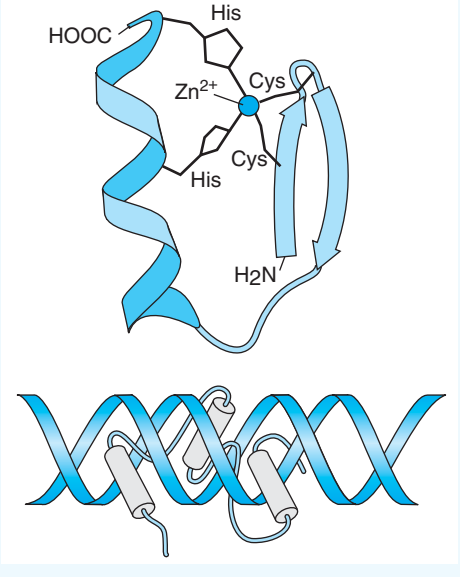
Protein sequence can be inferred from DNA sequence, although the degeneracy of the genetic code will result in several possible protein sequences for a given DNA sequence. Many databases are available on the frequency of codon usage in various organisms.[32-34] These can be used to predict the correct amino acid sequence.

of organization is the **secondary structure** of the protein. Some proteins, especially structural proteins, consist almost entirely of alpha helices or beta sheets. Globular proteins have varying amounts of alpha helix and beta sheets.

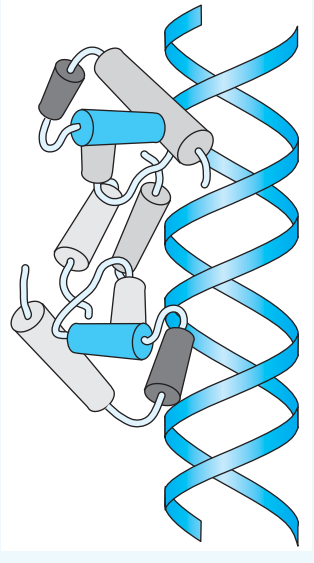
The secondary structures of proteins are further folded and arranged into a **tertiary structure**. Tertiary structure is important for protein function. If a protein loses its tertiary structure, it is **denatured**. Mutations in DNA that substitute different amino acids in the primary structure

Advanced Concepts

Specialized secondary structures can identify functions of proteins. **Zinc finger** motifs are domains frequently found in proteins that bind to DNA. These structures consist of two beta sheets followed by an alpha helix with a stabilizing zinc atom. There are three types of zinc fingers, depending on the arrangement of cysteine residues in the protein sequence. Another example of specialized secondary structure is the **leucine zipper**, also found in transcription factors.[35] This conserved sequence has a leucine or other hydrophobic residue at each seventh position for approximately 30 amino acids. The sequence is arranged in an alpha helical conformation such that the leucine side chains radiate outwardly to facilitate association with other peptides of similar structure. Because other amino acids besides leucine can participate in this interaction, the term basic zipper, or **bZip**, has been used to describe this type of protein structure. Another similar structure found in transcriptional regulators is the **helix loop helix.**[36] This motif consists of basic amino acids that bind consensus DNA sequences (CANNTG) of target genes. This structure is sometimes confused with the **helix turn helix**. The helix turn helix is two alpha helices connected by a short sequence of amino acids. This structure can easily fit into the major groove of DNA.



3-1 Zinc finger motif of the Sp1 protein, a eukaryotic transcription regulator. The side chains of histidine (H) and cysteine (C), part of the zinc finger amino acid sequence, C-$X_{2\text{-}4}$-C-X_3-F-X_5-L-X_2-H-X_3-H (~23 amino acids), bind a Zn atom in the active protein. Sp1 binds DNA in the regulatory region of genes. Note: For single-letter amino acid code, see Figure 3-7. *X* in this consensus denotes any amino acid.



3-2 The lambda repressor, a transcription factor of the bacteriophage lambda, has a helix turn helix motif. One of each of the helices fits into the major groove of the DNA. Lambda repressor prevents transcription of genes necessary for active growth of the bacteriophage leading to host cell lysis.

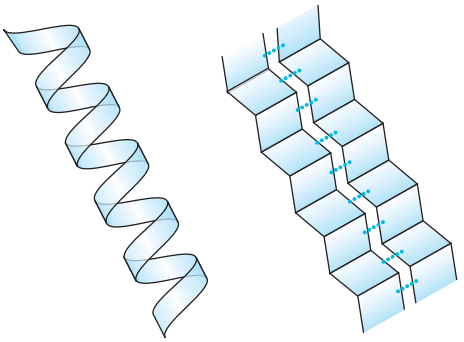


Figure 3-5 Secondary structure of proteins includes the alpha helix (A) and the beta pleated sheet (B). The ribbon-like structures in the pictures are composed of chains of amino acids hydrogen-bonded through their side chains.

can also alter tertiary structure. Denatured or improperly folded proteins are not functional. Proteins are also denatured by heat, e.g., the albumin in egg white, or by conformations forced on innocuous peptides by infectious **prions**. Aggregations of prion-induced aberrantly folded proteins cause transmissible spongiform encephalopathies such as Creutzfeldt-Jakob disease and bovine spongiform encephalitis (mad cow disease).

As previously mentioned, proteins often associate with other proteins in order to function. Two proteins bound together to function form a **dimer**, three a **trimer**, four a **tetramer**, and so forth. Proteins that work together in this way are called **oligomers**, each component protein being a **monomer**. This is the **quaternary structure** of proteins. The combinatorial nature of protein function may account for genetic complexity of higher organisms without concurrent increase in gene number.

Proteins are classified according to function as enzymes and transport, storage, motility, structural, defense, or regulatory proteins. Enzymes and transport, defense, and regulatory proteins are usually **globular** in nature, making them soluble and allowing them to diffuse freely across membranes. Structural and motility proteins, such as myosin, collagen, and keratin, are **fibrous** and insoluble.

In contrast to simple proteins that have no other components except amino acids**, conjugated proteins** do have components other than amino acids. The nonprotein component of a conjugated protein is the **nonprotein prosthetic group**. Examples of conjugated proteins are those covalently attached to lipids (**lipoproteins**), e.g., low density lipoproteins; sugars (**glycoproteins**), e.g., mucin in saliva; and metal atoms (**metalloproteins**), e.g., ferritin. One of the most familiar examples of a conjugated protein is hemoglobin. Hemoglobin is a tetramer with four Fe^{2+}-containing heme groups, one covalently attached to each monomer.

Genes and the Genetic Code

A **gene** is defined as the ordered sequence of nucleotides on a chromosome that encodes a specific functional product. A gene is the fundamental physical and functional unit of inheritance. The physical definition of a gene was complicated in early texts because of the methods used to define units of genetic inheritance. Genes were first studied by tracking mutations that took away their function. A gene was considered that part of the chromosome responsible for the function affected by mutation. Genes were not delineated well in terms of their physical size but were mapped relative to each other based on the frequency of recombination between them.

In the early 1960s, Seymour Benzer[6,7] used the T4 bacteriophage to investigate the gene more closely. By mixing phage with several different phenotypes, he could observe the complementation of one mutant for another. Benzer could distinguish mutations that could complement each other, even though they affected the same phenotype and mapped to the same locus. He determined that these were mutations in different places within the same gene. He organized many mutants into a series of sites along the linear array of the phage chromosome so that he could structurally define the gene as a continuous linear span of genetic material.

We know now that a gene contains not only **structural sequences** that code for an amino acid sequence but also **regulatory sequences** that are important for the regulated expression of the gene (Fig. 3-6). Cells expend much energy to coordinate protein synthesis so that the proper proteins are available at specific times and in specific amounts. Loss of this controlled expression will result in an abnormal phenotype, even though there may be no mutation in the structural sequence of the gene. Lack of appreciation for the importance of proximal and distal regulatory sequences was another source of confusion in early efforts to define a gene. Regulatory effects

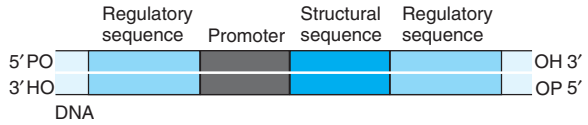


Figure 3-6 A gene contains not only structural (coding) sequences but also sequences important for regulated transcription of the gene. These include the promoter, where RNA polymerase binds to begin transcription, and regulatory regions, where transcription factors and other regulatory factors bind to stimulate or inhibit transcription by RNA polymerase.

and the interaction between proteins still challenge interpretation of genetic analyses in the clinical laboratory.

The Genetic Code

The nature of a gene was further clarified with the deciphering of the **genetic code** by Francis Crick, Marshall Nirenberg, Philip Leder, Gobind Khorana, and Sydney Brenner.[8-10] The genetic code is not information in itself but is a dictionary to translate the four-nucleotide sequence information in DNA to the 20–amino acid sequence information in proteins.

Historical Highlights

The interesting history of the breaking of the genetic code began with a competitive scramble. A physicist and astronomer, George Gamow, organized a group of scientists to concentrate on the problem. They called themselves the RNA Tie Club. Each of the 20 members wore a tie emblazoned with a depiction of RNA and a pin depicting a different amino acid. The club members were William Astbury, Oswald Avery, Sir William Laurence Bragg, Erwin Chargaff, Martha Chase, Robert Corey, Francis Crick, Max Delbruck, Jerry Donohue, Rosalind Franklin, Bruce Fraser, Sven Furgerg, Alfred Hershey, Linus Pauling, Peter Pauling, Max Perutz, J.T. Randall, Verner Schomaker, Alexander R. Todd, James Watson, and Maurice Wilkins. This group met regularly during the 1950s but did not exclusively break the genetic code.

Early on, scientists had surmised the triplet nature of the code based on mathematical considerations. It was reasoned that the smallest set of 4 possible letters that would yield enough unique groups to denote 20 different amino acids was 3. A 1-nucleotide code could only account for 4 different amino acids, whereas a 2-nucleotide code would yield just 16 different possibilities. A 3-nucleotide code would give 64 different possibilities, enough to account for all 20 amino acids.

The next challenge was to decipher the triplet code and to prove its function. The simplest way to prove the code would have been to determine the sequence of nucleotides in a stretch of DNA coding for a protein and compare it with the protein sequence. In the early 1960s, protein sequencing was possible, but only limited DNA sequencing was available. Marshall Nirenberg made the initial attempts at the code by using short synthetic DNA sequences to support protein synthesis in a cell-free extract of *Escherichia coli*. In each of 20 tubes he mixed a different radioactive amino acid, cell lysate from *E. coli,* and an RNA template. In the first definitive experiment, the input template was a polymer of uracil, UUUUUUU…. If the input template supported synthesis of protein, the radioactive amino acid would be incorporated into the protein and the radioactivity detected in a precipitable protein extract from the mixture. On May 27, 1961, Nirenberg measured radioactive protein levels from all but 1 of the 20 vials at around 70 counts/mg. The vial containing phenylalanine yielded protein of 38,000 counts/mg. After the first demonstration of success of this strategy, other templates were tested. Each synthetic nucleic acid incorporated different amino acids, based on the composition of bases in the RNA sequence. Codes for phenylalanine (UUU), proline (CCC), lysine (AAA), and glycine (GGG) were soon deduced from the translation of nucleic acids synthesized from a single nucleotide population. More of the code was indirectly deduced using mixtures of nucleotides at different proportions. For instance, an RNA molecule synthesized from a 2:1 mixture of U and C polymerized mostly phenylalanine and leucine into protein. Similar tests with other nucleotide mixtures resulted in distinct amino acid incorporations. Although each RNA molecule in these tests was of known composition of nucleotides, the exact order of nucleotides in the triplet was not known.

Nirenberg and Leder used another technique to get at the basic structure of the code. They observed binding of

specific amino acids to RNA triplets in ribosome-tRNA mixtures. By noting which triplet/amino acid combination resulted in binding of the amino acid to ribosomes, they were able to assign 50 of the 64 triplets to specific amino acids.

Meanwhile, Gobind Khorana had developed a system to synthesize longer polymers of known nucleotide sequence. With polynucleotides of repeated sequence, he could predict and then observe the peptides that would come from the known sequence. For example, a polymer consisting of two bases such as ...UCUCUCUCUCUCUC... was expected to code for a peptide of two different amino acids, one coded for by UCU and one by CUC. This polymer yielded a peptide with the sequence ...Ser-Leu-Ser-Leu.... This experiment did not tell which triplet coded for which amino acid, but combined with the results from Nirenberg and Leder, the UCU was assigned to serine and CUC to leucine.

By 1965, all 64 triplets, or **codons**, were assigned to amino acids (Fig. 3-7). Once the code was confirmed, specific characteristics of it were apparent. The code is redundant, so that all but two amino acids (methionine and tryptophan) are coded for by more than one codon. Triplets coding for the same amino acid are similar, mostly differing only in the third base of the triplet. Crick first referred to this as **wobble** in the third position.[11] Wobble is also used to describe movement of the base in the third position of the triplet to form novel pairing between the carrier tRNA and the mRNA template during protein translation. Recent investigations have revealed that wobble may affect the severity of disease phenotype.[12]

All amino acids except leucine, serine, and arginine are selected by the first two letters of the genetic code. The first two letters, however, do not always specify unique amino acids. For example, CA starts the code for both histidine and glutamine. Three codons, UAG, UAA, and UGA, that terminate protein synthesis are termed **nonsense** codons. UAG, UAA, and UGA were named **amber**, **ocher**, and **opal**, respectively, when they were first defined in bacterial viruses.

The characteristics of the genetic code have consequences for molecular analysis. Mutations or changes in the DNA sequence will have different effects on phenotype depending on the resultant changes in the amino acid sequence. Accordingly, mutations range from silent

Second position of codon

First position	U	C	A	G	Third position
U	UUU, UUC Phenylalanine UUA, UUG Leucine	UCU, UCC, UCA, UCG Serine	UAU, UAC Tyrosine UAA Ter (end) UAG Ter (end)	UGU, UGC Cysteine UGA Ter (end) UGG Tryptophan	U C A G
C	CUU, CUC, CUA, CUG Leucine	CCU, CCC, CCA, CCG Proline	CAU, CAC Histidine CAA, CAG Glutamine	CGU, CGC, CGA, CGG Arginine	U C A G
A	AUU, AUC, AUA Isoleucine AUG Methionine	ACU, ACC, ACA, ACG Threonine	AAU, AAC Asparagine AAA, AAG Lysine	AGU, AGC Serine AGA, AGG Arginine	U C A G
G	GUU, GUC, GUA, GUG Valine	GCU, GCC, GCA, GCG Alanine	GAU, GAC Aspartic acid GAA, GAG Glutamic acid	GGU, GGC, GGA, GGG Glycine	U C A G

■ Figure 3-7 The genetic code. Codons are read as the nucleotide in the left column, then the row at the top, and then the right column. Note how there are up to six codons for a single amino acid. Only methionine and tryptophan have a single codon. Note also the three termination codons (ter), TAA, TAG, and TGA.

Advanced Concepts

The UGA codon also codes for selenocysteine. Selenoproteins have UGA codons in the middle of their coding regions. In the absence of selenium, protein synthesis stops prematurely in these genes.

to drastic in terms of their effects on phenotype. This will be discussed in more detail in later chapters.

An interesting observation about the genetic code is that, with limited exceptions, the repertoire of amino acids is limited to 20 in all organisms, regardless of growing environments. Thermophilic and cryophilic organisms adapt to growth at 100°C and freezing temperatures, respectively, not by using structurally different amino acids but by varying the combinations of the naturally occurring amino acids. As will be discussed in the next section, cells have strict control and editing systems to protect the genetic code and avoid incorporation of unnatural amino acids into proteins. Recent studies have shown that it is possible to manipulate the genetic code to incorporate modified amino acids.[13,14] The ability to introduce chemically or physically reactive sites into proteins in vivo has significant implications in biotechnology.

Translation

Amino Acid Charging

After transcription of the sequence information in DNA to RNA, the transcribed sequence must be transferred into proteins. Through the genetic code, specific nucleic acid sequence is translated to amino acid sequence and, ultimately, to phenotype.

Protein synthesis starts with activation of the amino acids by covalent attachment to tRNA, or **tRNA charging**, a reaction catalyzed by 20 **aminoacyl tRNA synthetases**. The Mg^{++}-dependent charging reaction was first described by Hoagland and Zamecnik, who observed that amino acids incubated with ATP and the cytosol fraction of liver cells became attached to heat-soluble RNA (tRNA).[15] The reaction takes place in two steps. First, the amino acid is activated by addition of AMP:

$$\text{amino acid} + \text{ATP} \rightarrow \text{aminoacyl-AMP} + \text{PPi}$$

Second, the activated amino acid is joined to the tRNA:

$$\text{aminoacyl-AMP} + \text{tRNA} \rightarrow \text{aminoacyl-tRNA} + \text{AMP}$$

The product of the reaction is an ester bond between the 3′ hydroxyl of the terminal adenine of the tRNA and the carboxyl group of the amino acid.

There are 20 amino acyl tRNA synthetases, one for each amino acid. Designated into two class I and class II synthetases, these enzymes interact with the minor or major groove of the tRNA acceptor arm, respectively. Both classes also recognize tRNAs by their anticodon sequences and amino acids by their side chains. Only the appropriate tRNA and amino acid will fit into its cognate synthetase (Fig. 3-8). An errant amino acid bound to the wrong synthetase will dissociate rapidly before any conformation changes and charging can occur. In another level of editing, mischarged aminoacylated tRNAs are hydrolyzed at the point of release from the enzyme. The fidelity of this system is such that mischarging is 10^3–10^6 less efficient than correct charging.[16]

Advanced Concepts

According to evolutionary theory, the genetic code has evolved over millions of years of selection. An interesting analysis was done to compare the natural genetic code shared by all living organisms with millions of other possible triplet codes (4 nucleotides coding for 20 amino acids) generated by computer.[37] The results showed that the natural code was significantly more resistant to damaging changes (mutations in the DNA sequence) compared with the other possible codes.

Protein Synthesis

Translation takes place on **ribosomes,** small ribonucleoprotein particles first observed by electron microscopy of animal cells. In the early 1950s, Zamecnik demonstrated by pulse labeling that these particles were the site of protein synthesis in bacteria.[17] There are about 20,000 ribosomes in an *E. coli* cell, making up almost 25% of the cell's dry weight. Ribosomal structure is similar in prokaryotes and eukaryotes (see Fig. 2-3). In prokaryotes, 70S ribosomes are assembled from a 30S small subunit and a 50S large subunit, in association with mRNA and initiating factors (S stands for sedimentation units in density gradient centrifugation, a method used to deter-

Advanced Concepts

Once the amino acid is esterified to the tRNA, it makes no difference in specificity of its addition to the protein. The fidelity of translation is now determined by the **anticodon** (complementary three bases to the amino acid codon) of the tRNA as an adaptor between mRNA and the growing protein. This association has been exploited by attaching amino acids synthetically to selected tRNAs. If amino acids attached are to tRNAs carrying anticodons to UAG, UAAm or UGA, the peptide chain will continue to grow instead of terminating at the stop codon. These tRNAs are called **suppressor tRNAs** as they can suppress point mutations that generate stop codons within a protein coding sequence.

mine sizes of proteins and protein complexes). The 30S subunit (1 million daltons) is composed of a 16S ribosomal RNA (rRNA) and 21 ribosomal proteins. The 50S subunit (1.8 million daltons) is composed of a 5S rRNA, a 23S rRNA, and 34 ribosomal proteins. Eukaryotic ribosomes are slightly larger (80S) and more complex with a 40S small subunit (1.3 million daltons) and a 60S subunit (2.7 million daltons). The 40S subunit is made up of an 18S rRNA and about 30 ribosomal proteins. The 60S subunit contains a 5S rRNA, a 5.8S rRNA, a 28S rRNA, and about 40 ribosomal proteins.

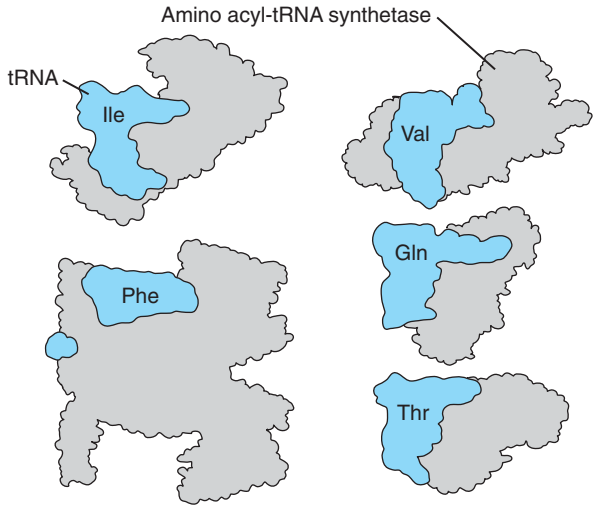


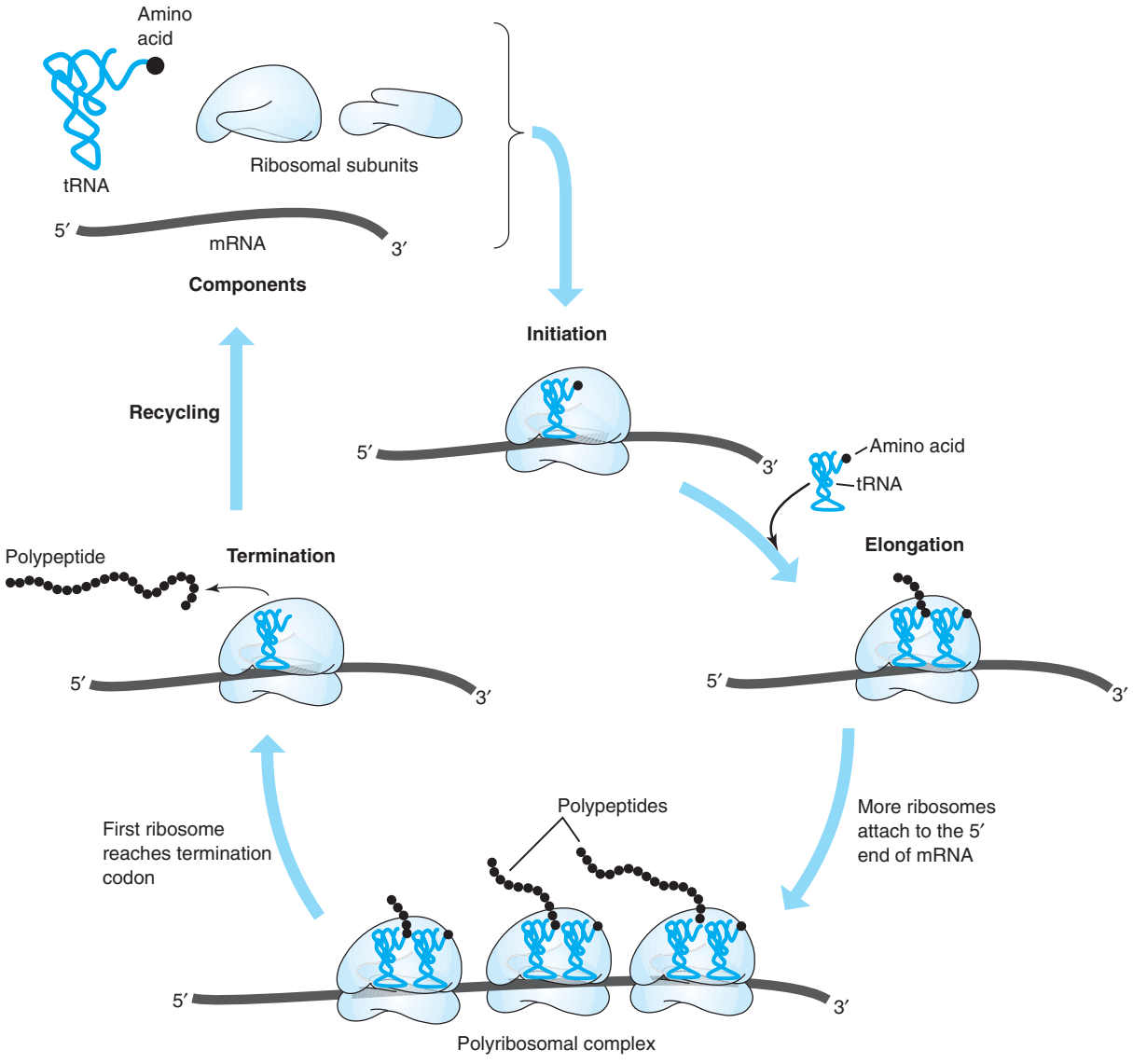
Figure 3-8 Five amino acyl tRNA synthetases. Each enzyme is unique for a tRNA (shown in green) and its matching amino acid. The specificity of these enzymes is key to the fidelity of translation.

Protein synthesis in the ribosome almost always starts with the amino acid methionine in eukaryotes and N-formylmethionine in bacteria, mitochondria, and chloroplasts. Initiating factors that participate in the formation of the ribosome complex differentiate the initiating methionyl tRNAs from those that add methionine internally to the protein.

In protein translation, the small ribosomal subunit first binds to initiation factor 3 (IF-3) and then to specific sequences near the 5′ end of the mRNA, the **ribosomal binding site**. This guides the AUG codon (the "start" codon) to the proper place in the ribosomal subunit. Another initiation factor, IF-2 bound to GTP and the initiating $tRNA^{Met}$ or $tRNA^{fMet}$, then joins the complex (Fig. 3-9). The large ribosomal subunit then associates coordinate with the hydrolysis of GTP and release of GDP and phosphate, IF-2 and IF-3. The resulting functional 70S or 80S ribosome is the **initiation complex**. In this complex, the $tRNA^{Met}$ or $tRNA^{fMet}$ is situated in the peptidyl site (**P site**) of the functional ribosome. $tRNA^{Met}$ or $tRNA^{fMet}$ can only bind to the P site in the ribosome, which is formed in combination by both ribosomal subunits. In contrast, all other tRNAs bind to an adjacent site, the aminoacyl site (**A site**) of the ribosome.

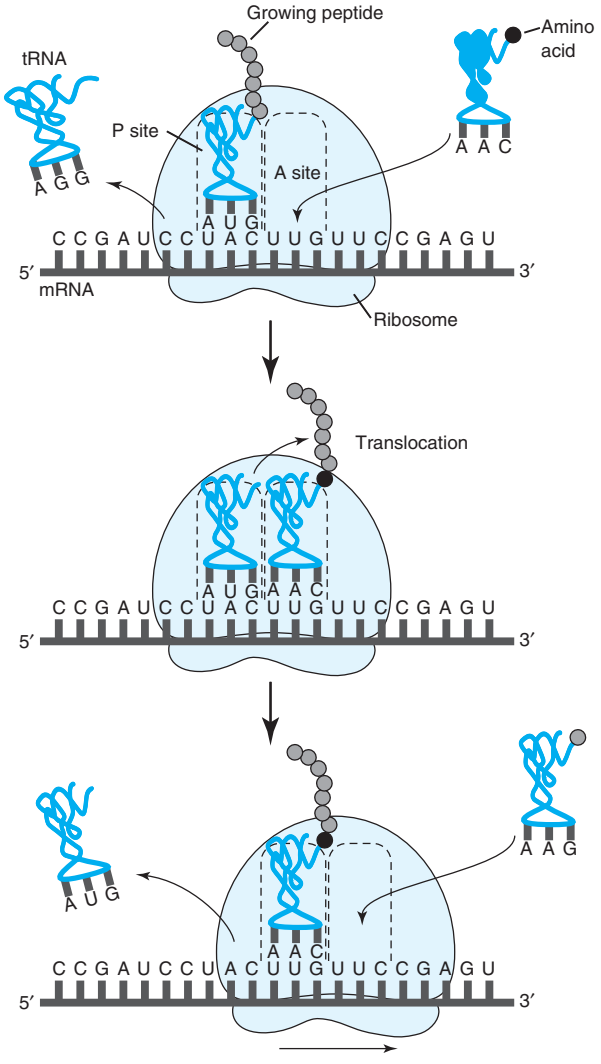
Synthesis proceeds in the elongation step where the tRNA carrying the next amino acid binds to the A site of the ribosome in a complex with elongation factor Tu (EF-Tu) and GTP (Fig. 3-10). The fit of the incoming tRNA takes place by recognition and then proofreading of the codon-anticodon base pairing. Hydrolysis of GTP by EF-Tu occurs between these two steps.[18] The EF-Tu-GDP is released, and the EF-Tu-GTP is regenerated by another elongation factor, EF-Ts. Although these interactions ensure the accurate paring of the first two codon positions, the pairing at the third position is not as stringent, which accounts for the wobble in the genetic code.[19]

The first peptide bond is formed between the amino acids in the A and P sites by transfer of the N-formylmethionyl group to the amino group of the second amino acid, leaving a dipeptidyl-tRNA in the A site. This step is catalyzed by an enzymatic activity in the large subunit,

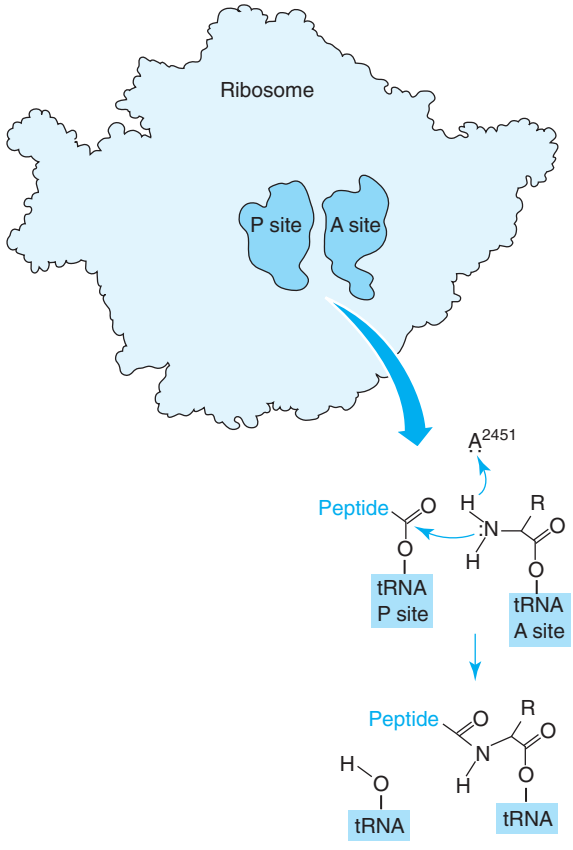


■ Figure 3-9 Assembly of the small ribosome subunit with mRNA and then the large ribosomal subunit and charged tRNA initiates RNA synthesis (initiation). Binding of charged tRNAs and formation of the peptide bond produce the growing polypeptide (elongation). Several ribosomes can simultaneously read a single mRNA (polyribosome complex). When the complex encounters a nonsense codon, protein synthesis stops (termination), and the components are recycled.

peptidyl transferase. This activity might be mediated entirely through RNA, as no proteins are in the vicinity of the active site of the ribosome where the peptide bond formation occurs.[20] After formation of the peptide bond, the ribosome moves, shifting the dipeptidyl-tRNA from the A site to the P site with the release of the "empty" tRNA from a third position, the **E site**, of the ribosome. This movement (translocation) of tRNAs across a distance of 20 angstroms from the A to the P site and 28 angstroms from the P to the E site requires elongation



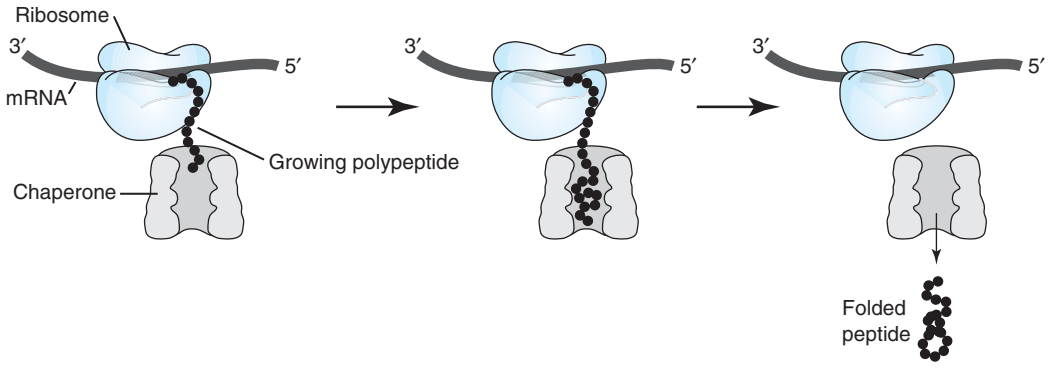
■ Figure 3-10 Incoming charged tRNAs bind to the A site of the ribosome, guided by matching codon-anticodon pairing. After formation of the peptide bond between the incoming amino acid and the growing peptide, the ribosome moves to the next codon in the mRNA, translocating the peptide to the P site and creating another A site for the next tRNA.



■ Figure 3-11 Protein synthesis (translation) as it takes place in the ribosome. The peptide bond is formed in an area between the large and small subunits of the ribosome. The ribozyme theory holds that the ribosome is an enzyme that functions through RNA and not protein. The close proximity of only RNA to this site is evidence for the ribozyme theory.

factor EF-G. As the ribosomal complex moves along the mRNA, the growing peptide chain is always attached to the incoming amino acid. Two GTPs are hydrolyzed to GDP with the addition of each amino acid. This energy-dependent translocation occurs with shifting and rotation of ribosomal subunits (Fig. 3-11).[21]

During translation, the growing polypeptide begins to fold into its mature conformation. This process is assisted by **molecular chaperones**.[22] These specialized proteins bind to the large ribosomal subunit, forming a hydrophobic pocket that holds the emerging polypeptide (Fig. 3-12). Chaperones apparently protect the hydrophobic regions of unfinished polypeptides until they can be safely associated inside the protein. In the absence of this activity, unfinished proteins might bind to each other and form nonfunctional aggregates. The DnaK protein of *E. coli* can also act as a chaperone by binding to the hydrophobic regions of the emerging polypeptide.[23]



■ Figure 3-12 Molecular chaperones catch the growing peptide as it emerges from the active site. The peptide goes through stages of holding (left), folding (center), and release (right). When the protein is completely synthesized and released from the ribosome, it should be in its folded state. This protects the nascent (growing) peptide from harmful interactions with other proteins in the cell before it has had an opportunity to form its protective and active tertiary structure.

Termination of the amino acid chain is signaled by one of the three nonsense, or termination, codons, UAA, UAG, or UGA, which are not charged with an amino acid. When the ribosome encounters a termination codon, termination, or **release factors** (R_1, R_2, and S in *E. coli*), causes hydrolysis of the finished polypeptide from the final tRNA, release of that tRNA from the ribosome, and dissociation of the large and small ribosomal subunits. In eukaryotes, termination codon–mediated binding of polypeptide chain release factors (eRF1 and eRF3) trigger hydrolysis of peptidyl-tRNA at the ribosomal peptidyl transferase center.[24,25]

E. coli can synthesize a 300–400 amino acid protein in 10–20 seconds. Because the protein takes on its secondary structure as it is being synthesized, it already has its final conformation when it is released from the ribosome.

In bacteria, translation and transcription occur simultaneously. In nucleated cells, the majority of translation occurs in the cytoplasm. Several lines of evidence, however, suggest that translation might also occur in the nucleus. One line of evidence is that nuclei contain factors required for translation.[26,27] Furthermore, isolated nuclei can aminoacylate tRNAs and incorporate amino acids into proteins. Another support for nuclear translation is that **nonsense-mediated decay (NMD)**, degradation of messenger RNAs with premature termination codons, was proposed to occur in mammalian nuclei. Further investigations, however, have shown that NMD may not occur in the nuclei of lower eukaryotes.[28] As in procaryotes, nuclear translation may require concurrent transcription.[29,30]

• STUDY QUESTIONS •

1. Indicate whether the following peptides are hydrophilic or hydrophobic.
   a. MLWILSS
   b. VAIKVLIL
   c. CSKEGCPN
   d. SSIQKNET
   e. YAQKFQGRT
   f. AAPLIWWA
   g. SLKSSTGGQ

2. Is the following peptide positively or negatively charged at neutral pH?
   GWWMNKCHAGHLNGVYYQGGTY

3. Consider an RNA template made from a 2:1 mixture of C:A. What would be the three amino acids most frequently incorporated into protein?

4. What is the peptide sequence encoded in AUAUAUAUAUAUAUA…?

5. Write the anticodons 5′ to 3′ of the following amino acids:
   a. L
   b. T
   c. M
   d. H
   e. R
   f. I

6. A protein contains the sequence LGEKKWCLRVNPKGLDESKDYLSLKSKYLLL. What is the likely function of this protein? (Note: see Box A3-4.)

7. A histone-like protein contains the sequence: PKKGSKKAVTKVQKKDGKKRKRSRK. What characteristic of this sequence makes it likely to associate with DNA? (Note: see the Box on p. 53.)

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SECTION 2

Common Techniques in Molecular Biology

Chapter 4

Lela Buckingham

Nucleic Acid Extraction Methods

OUTLINE

OBJECTIVES

- Compare and contrast organic, inorganic, and solid-phase approaches for isolating cellular and mitochondrial DNA.
- Note the chemical conditions in which DNA precipitates and goes into solution.
- Compare and contrast organic and solid-phase approaches for isolating total RNA.
- Distinguish between the isolation of total RNA with that of messenger RNA.
- Describe the gel-based, spectrophotometric, and fluorometric methods used to determine the quantity and quality of DNA and RNA preparations.
- Calculate the concentration and yield of DNA and RNA from a given nucleic acid preparation.

The purpose of extraction is to release the nucleic acid from the cell for use in subsequent procedures. Ideally, the target nucleic acid should be free of contamination with protein, carbohydrate, lipids, or other nucleic acid, i.e., DNA free of RNA or RNA free of DNA. The initial release of the cellular material is achieved by breaking the cell and nuclear membranes (cell lysis). Lysis must take place in conditions that will not damage the nucleic acid. Following lysis, the target material is purified, and then the concentration and purity of the sample can be determined.

Isolation of DNA

Although Miescher first isolated DNA from human cells in 1869,[1] the initial routine laboratory procedures for DNA isolation were developed from density gradient centrifugation strategies. Meselson and Stahl used such a method in 1958 to demonstrate semiconservative replication of DNA.[2] Later procedures made use of the differences in solubility of large chromosomal DNA, plasmids, and proteins in alkaline buffers. Large (>50 kbp) chromosomal DNA and proteins cannot renature properly when neutralized in acetate at low pH after alkaline treatment, forming large aggregates instead. As a result, they precipitate out of solution. The relatively small plasmids return to their supercoiled state and stay in solution. Alkaline lysis procedures were used extensively for extraction of 1–50–kb plasmid DNA from bacteria during the early days of recombinant DNA technology.

Preparing the Sample

Nucleic acid is routinely isolated from human, fungal, bacterial, and viral sources in the clinical laboratory (Table 4.1). The initial steps in nucleic acid isolation depends on the nature of the starting material.

Advanced Concepts

In surveying the literature, especially early references, the starting material for DNA extraction had to be noted because that determined which extraction procedure was used. Extraction procedures are often modified to optimize the yield of specific products. A procedure designed to yield plasmid DNA does not efficiently isolate chromosomal DNA and vice versa.

Nucleated Cells in Suspension

Depending on the type of clinical sample that is sent for analysis, the specimen may have to be pretreated to make nucleated cells available from which the nucleic acid will be extracted. For instance, white blood cells (WBCs) must be isolated from blood or bone marrow specimens. This is done by either differential density gradient centrifugation or differential lysis. For differential density gradient centrifugation, whole blood or bone marrow mixed with isotonic saline is overlaid with **Ficoll**.

Ficoll is a highly branched sucrose polymer that does not penetrate biological membranes. Upon centrifugation, the mononuclear WBCs (the desired cells for isolation of nucleic acid) settle into a layer in the Ficoll gradient that is below the less dense plasma components and above the polymorphonuclear cells and red blood cells (RBCs). The layer containing the mononuclear cells is removed from the tube and washed by at least two rounds of resuspension and centrifugation in saline before proceeding with the nucleic acid isolation procedure.

Another method used to isolate nucleated cells takes advantage of the differences in the osmotic fragility of RBCs and WBCs. Incubation of whole blood or bone marrow in hypotonic buffer or water will result in the lysis of the RBCs before the WBCs. The WBCs are then pelleted by centrifugation, leaving the empty RBC membranes (ghosts) and hemoglobin, respectively, in suspension and solution.

Tissue Samples

Fresh or frozen tissue samples must be dissociated before DNA isolation procedures can be started. Grinding the frozen tissue in liquid nitrogen, homogenizing the tissue, or simply mincing the tissue using a scalpel can disrupt whole tissue samples. Fixed embedded tissue has to be deparaffinized by soaking in xylene (a mixture of three isomers of dimethylbenzene). Less toxic xylene substitutes, such as Histosolve, Anatech Pro-Par, or ParaClear, are also often used for this purpose. After xylene treatment, the tissue is usually rehydrated by soaking it in decreasing concentrations of ethanol.

Microorganisms

Some bacteria and fungi have tough cell walls that must be broken to allow the release of nucleic acid. Several

Table 4.1 **Yield of DNA from Different Specimen Sources[11–18]**

	Specimen	Expected Yield*
Specimens adequate for analysis without DNA amplification		
	Blood† (1 mL, 3.5–10 × 10^6 WBCs/mL)	20–50 μg
	Buffy coat† (1 mL whole blood)	50–200 μg
	Bone marrow† (1 mL)	100–500 μg
	Cultured cells (10^7 cells)	30–70 μg
	Solid tissue‡ (1 mg)	1–10 μg
	Lavage fluids (10 mL)	2–250 μg
	Mitochondria (10-mg tissue, 10^7 cells)	1–10 μg
	Plasmid DNA, bacterial culture, (100-mL overnight culture)	350 μg–1 mg
	Bacterial culture (0.5 mL, 0.7 absorbance units)	10–35 μg
	Feces§ (1 mg; bacteria, fungi)	2–228 μg
Specimens adequate for analysis with DNA amplification		
	Serum, plasma, cerebrospinal fluid‖ (0.5 mL)	0.3–3 μg
	Dried blood (0.5-1–cm diameter spot)	0.04–0.7 μg
	Saliva (1 mL)	5–15 μg
	Buccal cells (1 mg)	1–10 μg
	Bone, teeth (500 mg)	30–50 μg
	Hair follicles#	0.1–0.2 μg
	Fixed tissue** (5-10 × 10–micron sections)	6–50 μg
	Feces†† (animal cells, 1 mg)	2–100 pg

*Yields are based on optimal conditions. Assays will vary in yield and purity of sample DNA.
†DNA yield will vary with WBC count.
‡DNA yield will depend on type and condition of tissue.
§Different bacterial types and fungi will yield more or less DNA.
‖ DNA yield will depend on degree of cellularity.
¶Dried blood yield from paper is less than from textiles.
#Mitochrondrial DNA is attainable from hair shafts.
**Isolation of DNA from fixed tissue is affected by the type of fixative used and the age and the preliminary handling of the original specimen.
††Cells in fecal specimens are subjected to digestion and degradation.

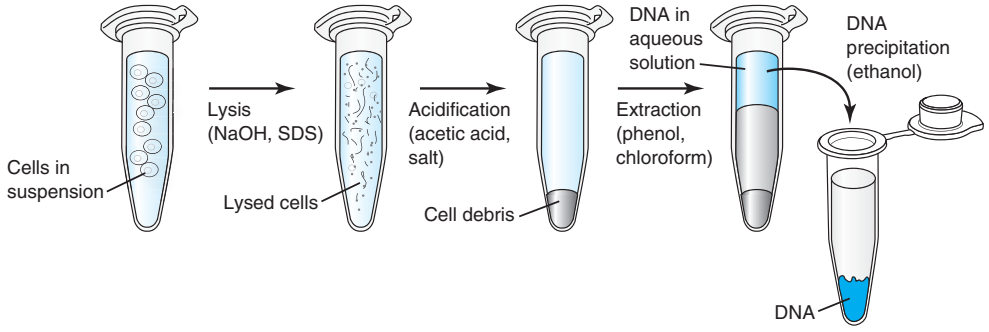
enzyme products, e.g., lyzozyme or zymolyase, that digest cell wall polymers are commercially available. Alternatively, cell walls can be broken mechanically by grinding or by vigorously mixing with glass beads. Gentler enzymatic methods are less likely to damage chromosomal DNA and thus are preferred for methods involving larger chromosomal targets as opposed to plasmid DNA.

Treatment with detergent (1% sodium dodecyl sulfate) and strong base (0.2 M NaOH) in the presence of Tris base, ethylenediaminetetraacetic acid (EDTA), and glucose can also break bacterial cell walls.

Boiling in 8% sucrose, 8% Triton X-100 detergent, Tris buffer, and EDTA after lysozyme treatment releases DNA that can be immediately precipitated with alcohol (see below). DNA extracted with NaOH or boiling procedures is denatured (single-stranded) and may not be suitable for methods such as restriction enzyme analysis that require double-stranded DNA. The advantage of these types of extraction is their speed and simplicity. Amplification methods will work with this type of DNA isolation.

Organic Isolation Methods

After release of DNA from the cell, further purification requires removal of contaminating proteins, lipids, carbohydrates, and cell debris. This is accomplished using a combination of high salt, low pH, and an organic mix-



■ Figure 4-1 General scheme of organic DNA isolation.

ture of phenol and chloroform. The combination readily dissolves hydrophobic contaminants such as lipids and lipoproteins, collects cell debris, and strips away most DNA-associated proteins (Fig. 4-1). Isolation of small amounts of DNA from challenging samples such as fungi can be facilitated by pretreatment with cetyltrimethylammonium bromide, a cationic detergent that efficiently separates DNA from polysaccharide contamination. To avoid RNA contamination, RNAse, an enzyme that degrades RNA, can be added at this point. Alternatively, RNAse may also be added to the resuspended DNA at the end of the procedure.

When phenol and chloroform are added to the hydrophilic cleared cell lysate, a biphasic emulsion forms. Centrifugation will settle the hydrophobic layer on the bottom, with the hydrophilic layer on top. Lipids and other hydrophobic components will dissolve in the lower hydrophobic phase. DNA will dissolve in the upper aqueous phase. Amphiphilic components, which have both hydrophobic and hydrophilic properties as well as cell debris, will collect as a white precipitate at the interface between the two layers.

The upper phase containing the DNA is collected, and the DNA is then precipitated using ethanol or isopropanol in a high concentration of salt (ammonium, potassium or sodium acetate, or lithium or sodium chloride). The ethyl or isopropyl alcohol is added to the upper phase solution at 2:1 or 1:1 ratios, respectively, and the DNA forms a solid precipitate.

The DNA precipitate is collected by centrifugation. Excess salt is removed by rinsing the pellet in 70% ethanol, centrifuging and discarding the ethanol supernatant, and then dissolving the DNA pellet in rehydration buffer, usually 10 mM Tris, 1 mM EDTA (TE), or water.

Inorganic Isolation Methods

Safety concerns in the clinical laboratory make the use of caustic reagents such as phenol undesirable. Methods of DNA isolation that do not require phenol extraction have,

Advanced Concepts

Ethanol and isopropanol are used for molecular applications. The ethanol is one of the general use formulas, reagent grade. Reagent-grade alcohol (90.25% ethanol, 4.75% methanol, 5% isopropanol) is **denatured**; that is, the ethanol is mixed with other components because pure 100% ethanol cannot be distilled. The isopropanol used is undenatured, or **pure**, as it is composed of 99% isopropanol and 1% water with no other components.

The choice of which alcohol to use depends on the starting material, the size and amount of DNA to be isolated, and the design of the method. Isopropanol is less volatile than ethanol and precipitates DNA at room temperature. Precipitation at room temperature reduces coprecipitation of salt. Also, compared with ethanol, less isopropanol is added for precipitation; therefore, isopropanol can be more practical for large-volume samples. For low concentrations of DNA, longer precipitation times at freezer temperatures may be required to maximize the amount of DNA that is recovered. An important consideration to precipitating the DNA at freezer temperatures is that the increased viscosity of the alcohol at low temperatures will require longer centrifugation times to pellet the DNA.

Advanced Concepts

Sometimes, DNA preparations are intended for long-term storage. The presence of the chelating agent EDTA protects the DNA from damage by DNAses present in the environment. EDTA is a component of TE buffer (10 mM Tris, 1 mM EDTA) and other resuspension buffers. The EDTA will also inhibit enzyme activity when the DNA is used in various procedures such as restriction enzyme digestion or polymerase chain reaction (PCR). One must be careful not to dilute the DNA too far so that large volumes (e.g., more than 10% of a reaction volume) of the DNA-EDTA solution are required. When DNA yield is low, as is the case with some clinical samples, it is better to dissolve it in water. More of this can be used in subsequent procedures without adding excess amounts of EDTA. Because the entire sample will be used for analysis, protection on storage is not a concern.

therefore, been developed and are used in many laboratories. Initially, these methods did not provide the efficient recovery of clean DNA achieved with phenol extraction; however, newer methods have proven to produce high-quality DNA preparations in good yields.

Inorganic DNA extraction is sometimes called "**salting out**" (Fig. 4-2). It makes use of low pH and high salt conditions to selectively precipitate proteins, leaving the DNA in solution. The DNA can then be precipitated as described above using isopropanol pelleted and resuspended in TE buffer or water.

Advanced Concepts

Precipitation of the DNA excludes hydrophilic proteins, carbohydrates, and other residual contaminants still present after protein extraction. In addition, the concentration of the DNA can be controlled by adjusting the buffer or water volume used for resuspension of the pellet.

Solid-Phase Isolation

More rapid and comparably effective DNA extraction can be performed using solid matrices to bind and wash the DNA. Silica-based products were shown to effectively bind DNA in high salt conditions.[3] Many variations on this procedure have been developed, including use of diatomaceous earth as a source of silica particles.[4] More modern systems can be purchased with solid matrices in the form of columns or beads. Columns come in various sizes, depending on the amount of DNA to be isolated. Columns used in the clinical laboratory are usually small "**spin columns**" that fit inside microcentrifuge tubes. These columns are commonly used to isolate viral and bacterial DNA from serum, plasma, or cerebrospinal fluid. They are also used routinely for isolation of cellular DNA in genetics and oncology. Preparation of samples for isolation of DNA on solid-phase media starts with cell lysis and release of nucleic acids, similar to organic and inorganic procedures (Fig. 4-3). Specific buffers are used to lyse bacterial, fungal, or animal cells. Buffer systems designed for specific applications (e.g., bacterial cell lysis or human cell lysis) are commercially available.

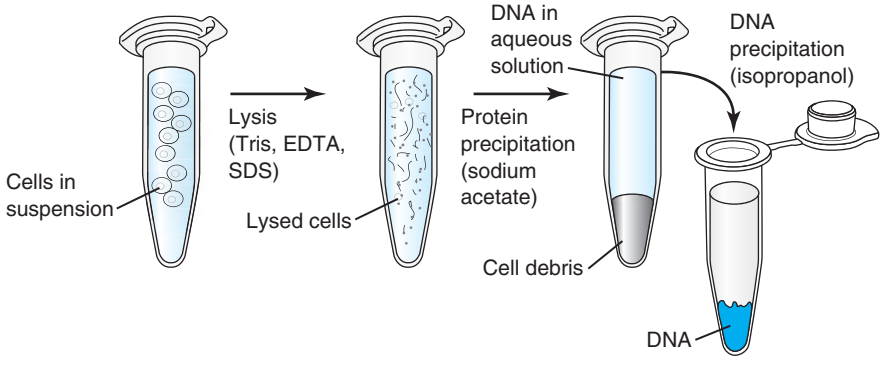


Figure 4-2 Inorganic DNA isolation.

Advanced Concepts

Alkaline lysis can be used to specifically select for plasmid DNA because chromosomal DNA will not renature properly upon neutralization and precipitate. The denatured chromosomal DNA and protein can be removed by centrifugation before the supernatant containing plasmid DNA is applied to the column.

Advanced Concepts

Solid matrices conjugated to specific sequences of nucleic acid can also be used to select for DNA containing complementary sequences by hybridization. After removal of noncomplementary sequences, the DNA can be eluted by heating the matrix or by breaking the hydrogen bonds chemically.

For solid-phase separation, the cell lysate is applied to a column in high salt buffer, and the DNA in solution adsorbs to the solid matrix. After the immobilized DNA is washed with buffer, the DNA is eluted in a specific volume of water, TE, or other low salt buffer. The washing solutions and the eluant can be drawn through the column by gravity, vacuum, or centrifugal force. DNA absorbed to magnetic beads is washed by suspension of the beads in buffer and collection of the beads using a magnet applied to the outside of the tube while the buffer is aspirated or poured off. The DNA IQ system (Promega) uses a magnetic resin that holds a specific amount of DNA (100 ng). When the DNA is eluted in 100 μL, the DNA concentration is known, 1 ng/μL, and ready for analysis.

Solid-phase isolation is the methodology employed for several robotic DNA isolation systems such as Roche MagnaPure and Qiagen BioRobot, which use magnetized glass beads or membranes to bind DNA. These systems are finding increased use in clinical laboratories for automated isolation of DNA from blood, tissue, bone marrow, plasma, and other body fluids. A measured amount of sample, e.g., 200–400 μL of whole blood or 10–50 mg of tissue, in sample tubes is placed into the instrument along with cartridges or racks of tubes containing the reagents used for isolation. Reagents are formulated in sets depending on the type and amount of starting material. The instrument is then programmed to lyse the cells and isolate and elute the DNA automatically.

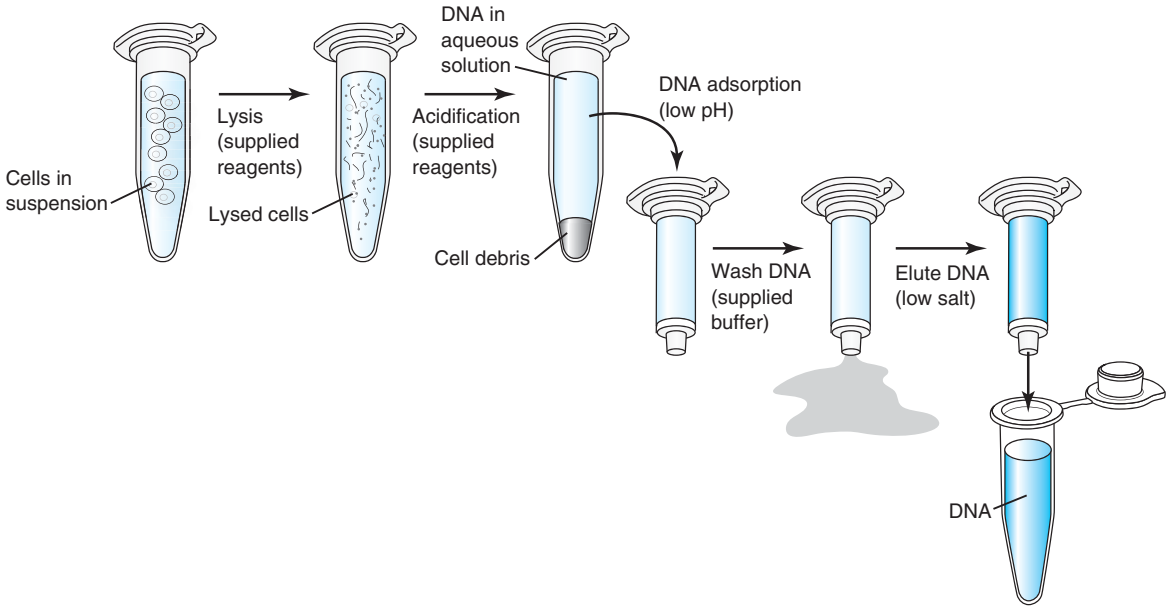


Figure 4-3 Isolation of DNA on solid media.

Crude Lysis

Although high-quality DNA preparations are tantamount to successful procedures, there are circumstances that either preclude or prohibit extensive DNA purification. These include screening large numbers of samples by simple methods (e.g., electrophoresis with or without restriction enzyme digestion and some amplification procedures), isolation of DNA from limited amounts of starting material, and isolation of DNA from challenging samples such as fixed, paraffin-embedded tissues. In these cases, simple lysis of cellular material in the sample will yield sufficiently useful DNA for amplification procedures.

Proteolytic Lysis of Fixed Material

Simple screening methods are mostly used for research purposes in which large numbers of samples must be processed. This is usually not done in the clinical laboratory. In contrast, the analysis of paraffin samples is frequently performed in the clinical laboratory. Fixed tissue is more conveniently accessed in the laboratory and may sometimes be the only source of patient material. Thin sections are usually used for analysis, although sectioning is not necessary with very small samples such as needle biopsies. Paraffin-embedded specimens must be dewaxed with xylene or other agents and then rehydrated before nucleic acid isolation. For some tests, such as somatic mutation analyses, a separate stained serial section can be examined microscopically to identify tumor cells. The identifiable areas of tumor can then be isolated directly from the slide by simple scraping in buffer (microdissection) or laser capture (Fig. 4-4) and deposited into microcentrifuge tubes.

Before lysis, cells may be washed by suspension and centrifugation in saline or other isotonic buffer. Reagents used for cell lysis depend on the subsequent use of the DNA. For simple screens, cells can be lysed in detergents such as SDS or Triton. For use in PCR amplification (see Chapter 7), cells may be lysed in a mixture of Tris buffer and proteinase K. The proteinase K will digest proteins in the sample, lysing the cells and inactivating other enzymes. The released DNA can be used directly in the amplification reaction.

Extraction With Chelating Resin

Chelex is a cation-chelating resin that can be used for simple extraction of DNA.[5,6] A suspension of 10% chelex resin beads is mixed with specimen, and the cells are lyzed by boiling. After centrifugation of the suspension, DNA in the supernatant is cooled and may be further extracted with chloroform before use in amplification procedures. This method is most commonly used in forensic applications but may also be useful for purification of DNA from clinical samples and fixed, paraffin-embedded specimens.[7,8]

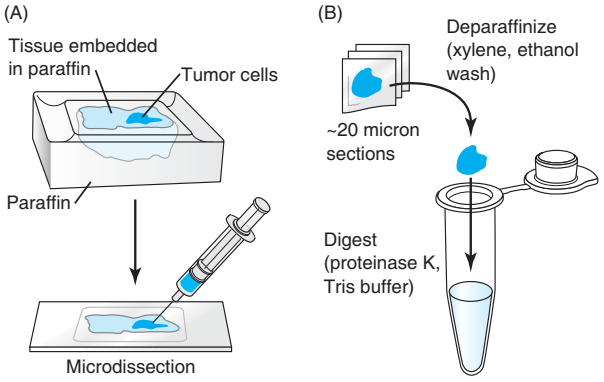


Figure 4-4 Crude extraction of DNA from fixed paraffin-embedded tissue. Selected regions of tissue are scraped from slides (A) and extracted (B).

Other Rapid Extraction Methods

With the advent of PCR, new and faster methods for DNA isolation have been developed. The minimal sample requirements of amplification procedures allow for the use of material previously not utilizable for analysis. Rapid lysis methods (produced by Sigma or Epicentre Technologies) and DNA extraction/storage cards (produced by Whatman) provide sufficiently clean DNA that can be used for amplification.

Isolation of Mitochondrial DNA

There are two approaches to the isolation of mitochondrial DNA from eukaryotic cells. One method is to first isolate the mitochondria by centrifugation. After cell preparations are homogenized by grinding on ice, the homogenate is centrifuged at low speed (700–2600 × g) to pellet intact cells, nuclei, and cell debris. The mitochondria can be pelleted from the supernatant in a second high-speed centrifugation (10,000–16,000 × g). The mitochondria can be lysed with detergent and the lysate treated with proteinase to remove protein contaminants. Mitochondrial DNA can then be precipitated with cold

Advanced Concepts

When homogenizing cells for isolation of mitochondria, care must be taken not to overgrind the tissue and dissociate the mitochondrial membranes. Grinding in alkaline buffers with reducing agents such as β-mercaptoethanol will protect the mitochondria during the isolation process. A high ionic strength buffer can also be used to selectively lyse the nuclear membranes.

ethanol and resuspended in water or appropriate buffers for analysis.

The second approach to mitochondrial DNA preparation is to isolate total DNA as described above. The preparation will contain mitochondrial DNA that can be analyzed within the total DNA background by hybridization or PCR.

Isolation of RNA

Working with RNA in the laboratory requires strict precautions to avoid sample RNA degradation. RNA is especially labile due to the ubiquitous presence of RNAses. These enzymes are small proteins that can renature, even after autoclaving, and become active. Unlike DNAses, RNAses must be eliminated or inactivated before isolation of RNA. They remain active at a wide range of temperatures (e.g., below -20°C and can renature even after heating).

It is useful to allocate a separate **RNAse-free** (RNF) area of the laboratory for storage of materials and specimen handling. Gloves must always be worn in the RNF area. Disposables (tubes, tips, etc.) that come in contact with the RNA should be kept at this location and never be touched by ungloved hands. Articles designated DNAse-free/RNF by suppliers may be used directly from the package. Reusable glassware is seldom used for RNA work. After cleaning, glassware must be baked for 4–6 hours at 400°C to inactivate the RNAses.

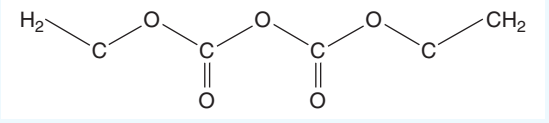
Total RNA

There are several types of naturally occurring RNA in prokaryotes and eukaryotes. (Refer to Chapter 2 for a more detailed discussion of RNA type and structure.) The most abundant (80%-90%) RNA in all cells is ribosomal RNA (rRNA). This RNA consists of two components, large and small, which are visualized by agarose gel electrophoresis (see Fig. 4-9). Depending on the cell type and conditions, the next most abundant RNA fraction (2.5%-5%) is messenger RNA (mRNA). This mRNA may be detected as a faint background underlying the rRNA detected by agarose gel electrophoresis. Transfer RNA and small nuclear RNAs are also present in the total RNA sample.

Advanced Concepts

Several chemical methods have been developed to inactivate or eliminate RNAses. Diethyl pyrocarbonate (**DEPC**) can be added to water and buffers (except for Tris buffer) to inactivate RNAses permanently. DEPC converts primary and secondary amines to carbamic acid esters. It can cross-link RNAse proteins through intermolecular covalent bonds, rendering them insoluble. Because of its effect on amine groups, DEPC will adversely affect Tris buffers. DEPC will also interact with polystyrene and polycarbonate and is not recommended for use on these types of materials.

Other RNAse inhibitors include vanandyl-ribonucleoside complexes, which bind the active sites of the RNAse enzymes, and macaloid clays, which absorb the RNAse proteins. Ribonuclease inhibitor proteins can be added directly to RNA preparations. These proteins form a stable noncovalent complex with the RNAses in solution. Some of these interactions require reducing conditions; therefore, dithiothreitol must be added in addition to the inhibitor.



Diethyl pyrocarbonate.

Extraction of Total RNA

Preparation of specimen material for RNA extraction differs in some respects than for DNA extraction. Reticulo-

Advanced Concepts

Specialized collection tubes are available for the isolation of RNA from whole blood (e.g., from Qiagen or Applied Biosystems). These tubes contain proprietary reagents that stabilize the intracellular RNA for several days at room temperature and longer at refrigerator temperature. The RNA can be isolated on a solid matrix.

cytes in blood and bone marrow samples are lysed by osmosis or separated from WBCs by centrifugation. When dissociating tissue, the sample should be kept frozen in liquid nitrogen or immersed in buffer that will inactivate intracellular RNAses. This is especially true for tissues such as pancreas that contain large amounts of innate RNAses. Bacterial and fungal RNA are also isolated by chemical lysis or by grinding in liquid nitrogen. Viral RNA can be isolated directly from serum or other cell-free fluids by means of specially formulated spin columns or beads. As most total RNA isolation methods cannot distinguish between RNA from microorganisms and those from host cells, cell-free material should be used for these isolations.

The cell lysis step for RNA isolation is done in detergent or phenol in the presence of high salt (0.2–0.5 M NaCl) or RNAse inhibitors. Guanidine thiocyanate is a strong denaturant of RNAses and can be used instead of high salt buffers. Strong reducing agents such as 2-mercaptoethanol can also be added during this step.

Once the cells are lysed, proteins can be extracted with phenol (Fig. 4-5). Acid phenol:chloroform:isoamyl alcohol (25:24:1) solution efficiently extracts RNA. Chloroform enhances the extraction of the nucleic acid by denaturing proteins and promoting phase separation. Isoamyl alcohol prevents foaming. For RNA, the organic phase must be acidic (ph 4–5). The acidity of the organic phase can be adjusted by overlaying it with buffer of the appropriate pH. In some isolation procedures, DNAse is added at the lysis step to eliminate contaminating DNA. Alternatively, RNAse-free DNAse also may be added directly to the isolated RNA at the end of the procedure. After phase separation, the upper aqueous phase containing the RNA is removed to a clean tube, and the RNA is precipitated by addition of two volumes of ethanol or one volume of isopropanol. Glycogen or yeast-transfer RNA may be added at this step as a carrier to aid RNA pellet formation. The RNA precipitate is then washed in 70% ethanol and resuspended in RNF buffer or water.

Solid-phase separation of RNA begins with similar steps as described above for organic extraction. The strong denaturing buffer conditions must be adjusted before application of the lysate to the column (Fig. 4-6). In some procedures, ethanol is added at this point. Some systems provide a filter column to remove particulate material before application to the adsorption column. As with DNA columns, commercial reagents are supplied with the columns to optimize RNA adsorption and washing on the silica-based matrix.

The lysate is applied to a column in high-salt chaotropic buffer, and the adsorbed RNA is washed with supplied buffers. DNAse can be added directly to the adsorbed RNA on the column to remove contaminating DNA. Washing solutions and the eluant can be drawn through the column by gravity, vacuum, or centrifugal force. Small columns of silica-based material that fit

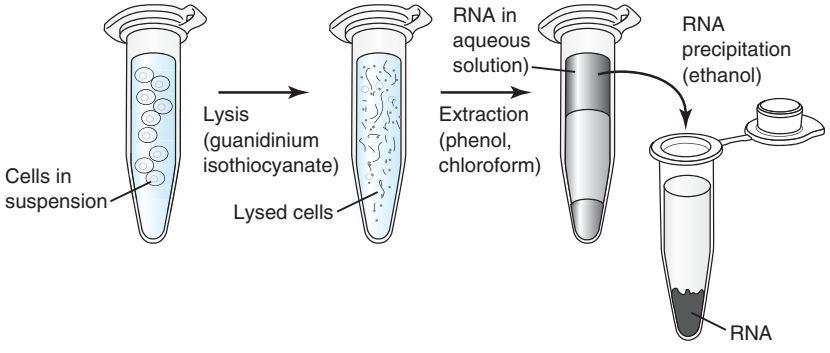
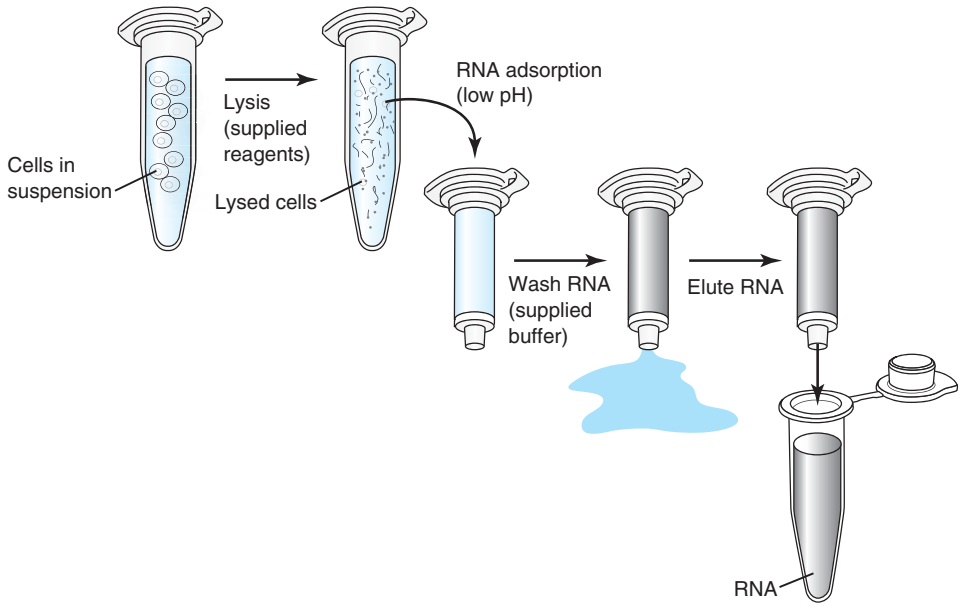


Figure 4-5 Organic extraction of total RNA.



■ Figure 4-6 Isolation of RNA on a solid matrix.

inside microfuge tubes (spin columns) are widely used for routine nucleic acid isolation from all types of specimens. The eluted RNA is usually of sufficient concentration and purity for direct use in most applications.

Generally, 1 million eukaryotic cells or 10–50 mg of tissue will yield about 10 μg of RNA. The yield of RNA from biological fluids will depend on the concentration of microorganisms or other target molecules present in the specimen (Table 4.2).

Isolation of polyA (messenger) RNA

As previously stated, approximately 80%–90% of total RNA is rRNA. Often the RNA required for analysis is mRNA, accounting for only about 2.5%–5% of the total RNA yield. The majority of mRNA consists of mRNA from highly expressed genes. Rare or single copy mRNA is, therefore, a very minor part of the total RNA isolation. To enrich the yield of mRNA, especially rare transcripts, protocols employing oligomers of thymine or uracil immobilized on a matrix resin column or beads are often used (Fig. 4-7). The polyT or polyU oligomers will bind the polyA tail found exclusively on mRNA. After washing away residual RNA, polyA RNA is eluted by washing the column with warmed, low-salt

Table 4.2 **Yield of RNA From Various Specimen Sources**[19-21]

Specimen	Expected Yield*
Blood† (1 mL, 3.5-10 × 10^6 WBCs/mL)	1–10 μg
Buffy coat† (1 mL whole blood)	5–10 μg
Bone marrow† (1 mL)	50–200 μg
Cultured cells‡ (10^7 cells)	50–150 μg
Buccal cells (1 mg)	1–10 μg
Solid tissue§ (1 mg)	0.5–4 μg
Fixed tissue‖ (1 mm^3)	0.2–3 μg
Bacterial culture¶ (0.5 mL, 0.7 absorbance units)	10–100 μg

*Specimen handling especially effects RNA yield. Isolation of polyA RNA will result in much lower yields. See text.
†RNA yield will depend on WBC count.
‡RNA yield will depend on type of cells and the conditions of cell culture.
§Liver, spleen, and heart tissues yield more RNA than brain, lung, ovary, kidney, or thymus tissues.
‖ Isolation of RNA from fixed tissue is especially affected by the type of fixative used and the age and the preliminary handling of the original specimen.
¶Different bacterial types and fungi will yield more or less RNA.

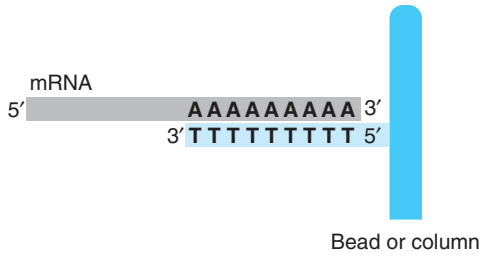


Figure 4-7 Oligo polythymine columns or beads bind the polyA tail of mRNA. The oligo can be poly uracil. Peptide nucleic acid dU or dT can also be used.

buffer containing detergent. With this approach, approximately 30–40 ng of mRNA can be obtained from 1 μg of total RNA.

There are limitations to the isolation of polyA RNA using oligo dT or dU. The efficiency of polyA and polyU binding is variable. Secondary structure (intrastrand or interstrand hydrogen bonds) in the target sample may compete with binding to the capture oligomer. Also, mRNAs with short polyA tails may not bind efficiently or at all. AT-rich DNA fragments might also bind to the column and not only compete with the desired mRNA target but also contaminate the final eluant. Potential digestion of the oligo-conjugated matrices precludes the use of DNase on the RNA before it is bound to the column. Treatment of the eluant with RNase-free DNase is possible, but the DNase should be inactivated if the mRNA is to be used in procedures involving DNA components. Furthermore, rRNA can copurify with the polyA RNA. The final purified product, then, is enriched in polyA RNA but is not a pure polyA preparation.

Measurement of Nucleic Acid Quality and Quantity

Laboratory analysis of nucleic acids produces variable results, depending on the quality and quantity of input material. This is an important consideration in the clinical laboratory, as test results must be accurately interpreted with respect to disease pathology. Consistent results require that run-to-run variation be minimized. Fortunately, measurement of the quality and quantity of DNA and RNA is straightforward.

Electrophoresis

DNA and RNA can be analyzed for quality by resolving an aliquot of the isolated sample on an agarose gel (Fig. 4-8; see Chapter 5 for a more detailed discussion of electrophoresis). Fluorescent dyes such as **ethidium bromide** or SybrGreen I bind specifically to DNA and are used to visualize the sample preparation. Ethidium bromide or SybrGreen II can be used to detect RNA. Less frequently, silver stain has been used to detect small amounts of DNA by visual inspection.

The appearance of DNA on agarose gels depends on the type of DNA isolated. A good preparation of plasmid DNA will yield a bright, moderate-mobility single band of supercoiled plasmid DNA with minor or no other bands that represent nicked or broken plasmid (see Fig. 4-8). High-molecular-weight genomic DNA should collect as a bright band with low mobility (near the top of the gel in Fig. 4-9). A high-quality preparation of RNA will yield two distinct bands of rRNA. The integrity of

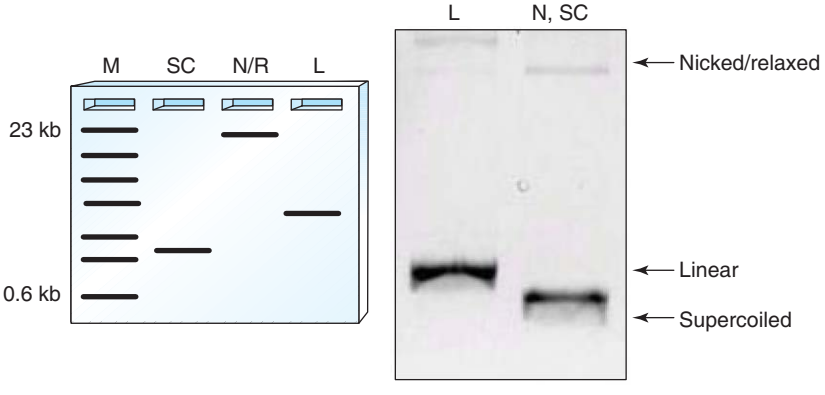
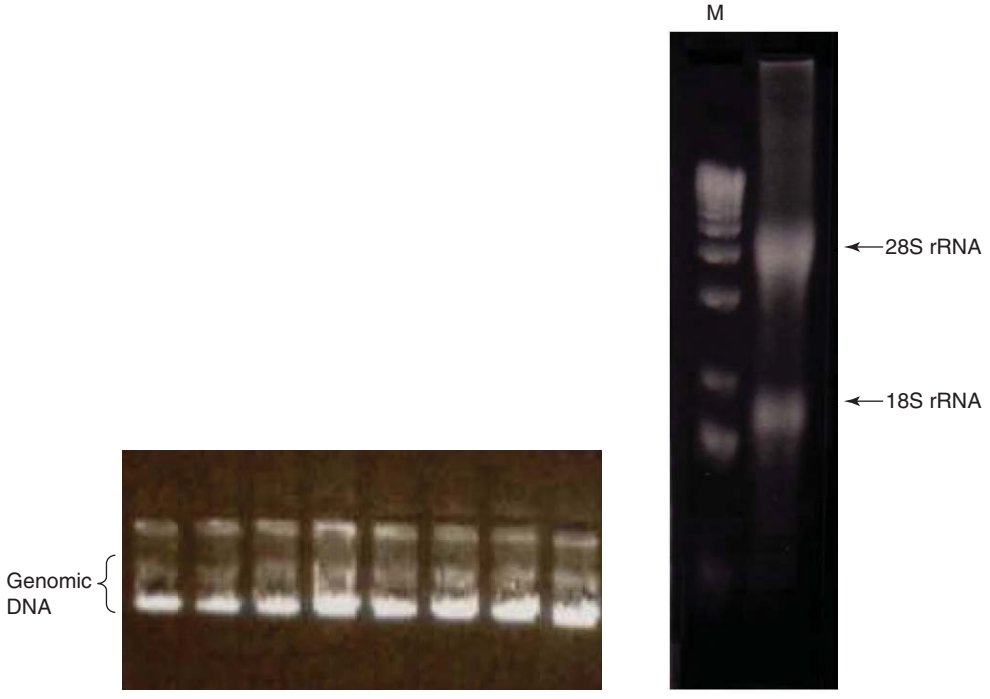


Figure 4-8 After agarose gel electrophoresis, compact supercoiled plasmid DNA (SC) will travel farther through the gel than nicked plasmid (N), which has single-strand breaks. Relaxed plasmid DNA (R) has double-strand breaks and will migrate according to its size, 23 kb in the drawing on the left. Linear (L) plasmids migrate according to the size of the plasmid. A gel photo shows a plasmid preparation. (nicked, N; supercoiled, SC; linear, L; relaxed, R; molecular weight markers, M)



■ Figure 4-9 Intact ethidium bromide–stained human chromosomal DNA (left) and total RNA (right) after agarose gel electrophoresis. High-quality genomic DNA runs as a tight smear close to the loading wells. High-quality total RNA appears as two rRNA bands (shown with molecular weight markers, M).

these bands is an indication of the integrity of the other RNA species present in the same sample. If these bands are degraded (smeared) or absent, the quality of the RNA in the sample is deemed unacceptable for use in molecular assays.

When fluorescent dyes are used, DNA and, less accurately, RNA can be quantitated by comparison of the fluorescence intensity of the sample aliquot run on the gel with that of a known amount of control DNA or RNA loaded on the same gel. Densitometry of the band intensities gives the most accurate measurement of quantity. For some procedures, estimation of DNA or RNA quantity can be made by visual inspection.

Spectrophotometry

Nucleic acids absorb light at 260 nm through the adenine residues. Using the Beer-Lambert Law, concentration can be determined from the **absorptivity constants** (50 for DNA, 40 for RNA). The relationship of concentration to absorbance is expressed as

$$A = \epsilon bc$$

where A = absorbance, ϵ = molar absorptivity (L/mol-cm), b = path length (cm), and c = concentration (mg/L). The absorbance at this wavelength is thus directly proportional to the concentration of the nucleic acid in the sample. Using the absorptivity as a conversion factor from optical density to concentration, one optical density unit (or absorbance unit) at 260 nm is equivalent to 50 mg/L (or 50 μg/mL) of DNA and 40 μg/mL of RNA. To determine concentration, multiply the spectrophotometer reading in absorbance units by the appropriate conversion factor. Phenol absorbs ultraviolet light at 270–275 nm, close to the wavelength of maximum absorption by nucleic acids. This means that residual phenol from organic isolation procedures can increase 260 readings, so phenol contamination must be avoided when measuring concentration at 260 nm.

Most DNA and RNA preparations are of sufficient concentration to require dilution before spectrophotometry in order for the reading to fall within the linear reading range (0.05–0.800 absorbance units, depending on the instrument). If the sample has been diluted before reading, the dilution factor must be included in the calculation of quantity. Multiply the absorbance reading by the conversion factor and the dilution factor to find the concentration of nucleic acid.

Example 1. A DNA preparation diluted 1:100 yields an absorbance reading of 0.200 at 260 nm. To obtain the concentration in μg/mL, multiply:

0.200 absorbance units × 50 μg/mL per absorbance unit × 100 = 1000 μg/mL

The **yield** of the sample is calculated using the volume of the preparation. If in the case illustrated above, the DNA was eluted or resuspended in a volume of 0.5 mL, the yield would be:

1000 μg/mL × 0.5 mL = 500 μg

Example 2. An RNA preparation diluted 1:10 yields an absorbance reading of 0.500 at 260 nm. The concentration is:

0.500 absorbance units × 40 μg/mL per absorbance unit × 10 = 200 μg/mL

The yield of the sample is calculated using the volume of the preparation. If in the case illustrated above, the DNA was eluted or resuspended in 0.2 mL, the yield would be:

200 μg/mL × 0.2 mL = 40 μg

Spectrophotometric measurements also indicate the quality of nucleic acid. Protein absorbs light at 280 nm through the tryptophane residues. The absorbance of the nucleic acid at 260 nm should be 1.6–2.00 times more than the absorbance at 280 nm. If the 260 nm/280 nm ratio is less than 1.6, the nucleic acid preparation may be contaminated with unacceptable amounts of protein and not of sufficient purity for use. Such a sample can be improved by reprecipitating the nucleic acid or repeating the protein removal step of the isolation procedure. It should be noted that low pH can affect the 260 nm/280 nm ratio. Somewhat alkaline buffers (pH 7.5) are recommended for accurate determination of purity. RNA affords a somewhat higher 260 nm/280 nm ratio, 2.0–2.3. A DNA preparation with a ratio higher than 2.0 may be contaminated with RNA. Some procedures for DNA analysis are not affected by contaminating RNA, in which case the DNA is still suitable for use. If, however, RNA may interfere or react with DNA detection components, RNase should be used to remove the contaminating RNA. Because it is difficult to detect contaminating DNA in RNA preparations, RNA should be treated with RNase-free DNase where DNA contamination may interfere.

Advanced Concepts

Newer models of ultraviolet spectrophotometers dedicated to nucleic acid analysis can be programmed to do the calculations described. The operator must enter the type of nucleic acid, the dilution factor, and the desired conversion factor. The instrument will automatically read the sample at both wavelengths and do the required calculations, giving a reading of concentration in μg/mL and a 260 nm/280 nm ratio.

Fluorometry

Fluorometry or fluorescent spectroscopy measures fluorescence related to DNA concentration in association with DNA-specific fluorescent dyes. Early methods used 3,5-diaminobenzoic acid 2HCl (DABA).[9] This dye combines with alpha methylene aldehydes (deoxyribose) to yield a fluorescent product. It is still used for the detection of nuclei and as a control for hybridization and spot integrity in microarray analyses.

More modern procedures use the DNA-specific dye **Hoechst 33258** {2-[2-(4-hydroxyphenyl)-(6-benzimidazol)]-6-(1-methyl-4-piperazyl)-benzimidazol/.3HCl}. This dye combines with adenine-thymine base pairs in the minor groove of the DNA double helix and is thus specific for intact double-stranded DNA. This binding specificity for A-T residues however, complicates measurements of DNA that have unusually high or low GC content. A standard measurement is required to determine concentration by fluorometry, and this standard must have GC content similar to that of the samples being measured. Calf thymus DNA (GC content = 50%) is often used as a standard for specimens with unknown DNA GC content. Fluorometric determination of DNA concentration using Hoechst dye is very sensitive (down to 200 ng DNA/mL).

PicoGreen and OliGreen (Molecular Probes, Inc.) are other DNA-specific dyes that can be used for fluorometric quantitation. Due to brighter fluorescence upon binding to double-stranded DNA, PicoGreen is more sensitive than Hoechst dye (detection down to 25 pg/mL concentrations). Like Hoechst dye, single-stranded DNA and RNA do not bind to PicoGreen. OliGreen is designed to bind to short pieces of single-stranded DNA (oligonucleotides). This dye will detect down to 100 pg/mL of single-stranded DNA. OliGreen will not fluoresce when bound to double-stranded DNA or RNA.

RNA may be measured in solution using SybrGreen II RNA gel stain.[10] Intensity of SyBrGreen II fluorescence is less with polyadenylated RNA than with total RNA by 20%–26%. The sensitivity of this dye is down to 2 ng/mL. SybrGreen II, however, is not specific to RNA and will bind and fluoresce with double-stranded DNA as well. Contaminating DNA must, therefore, be removed in order to get an accurate determination of RNA concentration.

Fluorometry measurements require calibration of the instrument with a known amount of standard before every run. For methods using Hoechst dye, the dye is diluted to a working concentration of 1 μg/mL in water. The dye is then mixed with the sample (usually a dilution of the sample). Once the dye and sample solution are mixed, fluorescence must be read within 2 hours because the dye/sample complex is stable only for approximately this amount of time. The fluorescence is read in a quartz cuvette. A programmed fluorometer will read out a concentration based on the known standard calibration.

Absorption and fluorometry readings may not always agree. First, the two detection methods recognize different targets. Single nucleotides do not bind to fluorescent dyes, but they can absorb ultraviolet light and affect spectrometric readings. Furthermore, absorption measurements do not distinguish between DNA and RNA, so contaminating RNA may be factored into the DNA measurement. RNA does not enhance fluorescence of the fluorescent dyes and is thus invisible to fluorometric detection. In fact, specific detection of RNA in the presence of DNA in solution is not yet possible. The decision which instrument to use is at the discretion of the laboratory. Most laboratories use spectrophotometry because the samples can be read directly without staining or mixing with dye. For methods that require accurate measurements of low amounts of DNA or RNA (in the 10–100–ng/mL range), fluorometry may be preferred.

• STUDY QUESTIONS •

DNA Quantity/Quality

1. Calculate the DNA concentration in μg/mL from the following information:
   a. Absorbance reading at 260 nm from a 1:100 dilution = 0.307
   b. Absorbance reading at 260 nm from a 1:50 dilution = 0.307
   c. Absorbance reading at 260 nm from a 1:100 dilution = 0.172
   d. Absorbance reading at 260 nm from a 1:100 dilution = 0.088

2. If the volume of the above DNA solutions was 0.5 mL, calculate the yield for a.–d.

3. Three DNA preparations have the following A_{260} and A_{280} readings:

Sample	OD_{260}	OD_{280}
1	*0.419*	*0.230*
2	*0.258*	*0.225*
3	*0.398*	*0.174*

   For each sample, based on the A_{260}/ A_{280} ratio, is each preparation suitable for further use? If not, what is contaminating the DNA?

RNA Quantity/Quality

1. Calculate the RNA concentration in μg/mL from the following information:
   a. Absorbance reading at 260 nm from a 1:100 dilution = 0.307
   b. Absorbance reading at 260 nm from a 1:50 dilution = 0.307
   c. Absorbance reading at 260 nm from a 1:100 dilution = 0.172
   d. Absorbance reading at 260 nm from a 1:100 dilution = 0.088

2. If the volume of the above RNA solutions was 0.5 mL, calculate the yield for a.–d.

3. An RNA preparation has the following absorbance readings:
   A_{260} = 0.208
   A_{280} = 0.096

   Is this RNA preparation satisfactory for use?

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Chapter 5

Lela Buckingham

Resolution and Detection of Nucleic Acids

OUTLINE

OBJECTIVES

- Explain the principle and performance of electrophoresis as it applies to nucleic acids.
- Compare and contrast the agarose and polyacrylamide gel polymers commonly used to resolve nucleic acids, and state the utility of each polymer.
- Explain the principle and performance of capillary electrophoresis as it is applied to nucleic acid separation.
- Give an overview of buffers and buffer additives used in electrophoretic separation, including the constituents, purpose, and importance.
- Describe the general types of equipment used for electrophoresis and how samples are introduced for electrophoretic separation.
- Compare and contrast pulse field gel electrophoresis and regular electrophoresis techniques with regards to method and applications.
- Compare and contrast detection systems used in nucleic acid applications.

Resolution and detection of nucleic acids are done in several ways. Gel and capillary electrophoresis are the most practical and frequently used methods. DNA can also be spotted and detected using specific hybridization probes, as will be described in Chapter 10.

Electrophoresis

Electrophoresis is the movement of molecules by an electric current. This can occur in solution, but it is practically done in a matrix to limit migration and contain the migrating material. Electrophoresis is routinely applied to the analysis of proteins and nucleic acids. Each phosphate group on a DNA polymer is ionized, making DNA a negatively charged molecule. Under an electric current, DNA will migrate toward the positive pole (anode). When DNA is applied to a macromolecular cage such as **agarose** or **polyacrylamide**, its migration under the pull of the current is impeded, depending on the size of the DNA and the spaces in the gel. Because each nucleotide has one negative charge, the charge-to-mass ratio of molecules of different sizes will remain constant. DNA fragments will therefore migrate at speeds inversely related to their size. Electrophoresis can be performed in tubes, slab gels, or capillaries. Slab gel electrophoresis can have either a horizontal or vertical format (Fig. 5-1).

Advanced Concepts

Double-stranded DNA and RNA are analyzed by native gel electrophoresis. The relationship between size and speed of migration can be improved by separating single-stranded nucleic acids; however, both DNA and RNA favor the double-stranded state. Unpaired, or **denatured**, DNA and RNA must, therefore, be analyzed in conditions that prevent the hydrogen bonding between complementary sequences. These conditions are maintained through a combination of formamide mixed with the sample, urea mixed with the gel, and/or heat-denaturing electrophoresis.

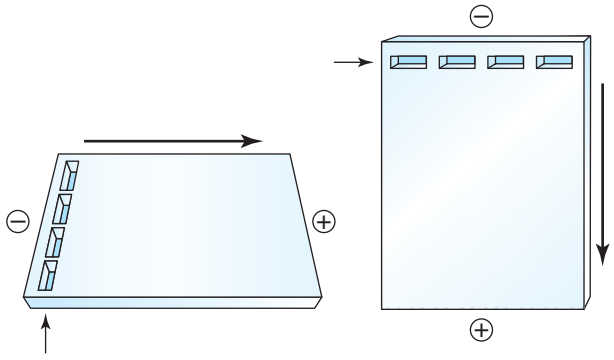


Figure 5-1 Horizontal (left) and vertical (right) gel electrophoresis. In both formats, sample is introduced into the gel at the cathode end (small arrows) and migrates with the current toward the anode.

Gel Systems

Gel matrices provide resistance to the movement of molecules under the force of the electric current. They prevent diffusion and reduce convection currents so that the separated molecules form a defined group, or "band." The gel can then serve as a support medium for analysis of the separated components. These matrices must be unaffected by electrophoresis, simple to prepare and amenable to modification. Agarose and polyacrylamide are polymers that meet these criteria.

Agarose Gels

Agarose is a polysaccharide polymer extracted from seaweed. It is a component of agar used in bacterial culture dishes. Agarose is a linear polymer of agarobiose, which consists of 1,3-linked-β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose (Fig. 5-2).

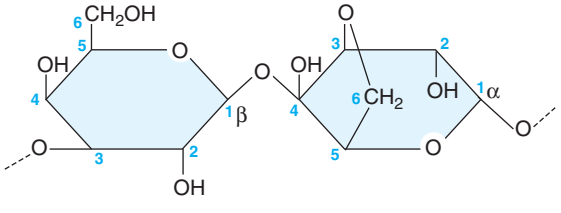


Figure 5-2 Agarobiose is the repeating unit of agarose.

Hydrated agarose gels in various concentrations, buffers, and sizes can be purchased ready for use. Alternatively, agarose can be purchased and stored in the laboratory in powdered form. For use, powdered agarose is suspended in buffer, heated, and poured into a mold. The concentration of the agarose dictates the size of the spaces in the gel and will, therefore, be determined by the size of DNA to be resolved (Table 5.1). Small pieces of DNA (50–500 bp) are resolved on more concentrated agarose gels, e.g., 2%–3% (Fig. 5-3). Larger fragments of DNA (2000–50,000) are best resolved in lower agarose concentrations, e.g., 0.5%–1%. Agarose concentrations above 5% and below 0.5% are not practical. High-concentration agarose will impede migration, whereas very low concentrations produce a weak gel with limited integrity.

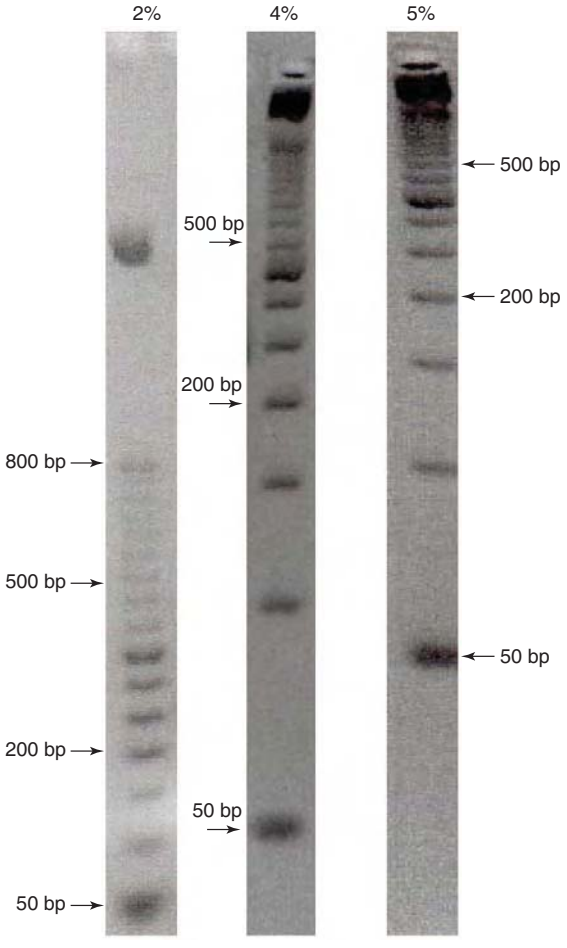


Figure 5-3 Resolution of double-stranded DNA fragments on 2%, 4%, and 5% agarose.

Advanced Concepts

The physical characteristics of the agarose gel can be modified by altering its polymer length and helical parameters. Several types of agarose are thus available for specific applications. The resolving properties differ in these preparations as well as the gelling properties. Low-melting agarose is often used for re-isolating resolved fragments from the gel. Other agarose types give better resolution of larger or smaller fragments. Modern agarose preparations are sufficiently pure to avoid problems such as **electroendosmosis**, a solvent flow toward one of the electrodes, usually the cathode (negative), in opposition to the DNA or RNA migration. This slows and distorts the migration of the samples, reducing resolution and smearing the bands.

Pulsed Field Gel Electrophoresis

Very large, i.e., 50,000–250,000 bp, pieces of DNA cannot be resolved efficiently by simple agarose electrophoresis. Even in the lowest concentrations of agarose, megabase fragments are too severely impeded for correct resolution (referred to as **limiting mobility**). Limiting mobility is reached when a DNA molecule can move only lengthwise through successive pores of the gel, a process called **reptation**.

For genomic-sized DNA molecules, pulses of current applied to the gel in alternating dimensions enhance

Table 5.1 **Choice of Agarose Concentration for DNA Gels*[10]**

Agarose Concentration (%)	Separation Range (size in bp)
0.3	5000–60,000
0.6	1000–20,000
0.8	800–10,000
1.0	400–8000
1.2	300–7000
1.5	200–4000
2.0	100–3000

*The table shows the range of separation for linear double-stranded DNA molecules in TAE agarose gels with regular power sources. Note that these values may be affected if another running buffer is used and if voltage is over 5 V/cm.

Table 5.2 **Choice of Polyacrylamide Concentration for DNA Gels*[10]**

Acrylamide Concentration (%)	Separation Range (size in bp)
3.5	100–1000
5.0	80–500
8.0	60–400
12.0	40–200
20.0	10–100

*The indicated figures are referring to gels run in TBE buffer. Voltages over 8 V/cm may affect these values.

migration. This process is called **pulsed field gel electrophoresis** (PFGE) (Fig. 5-4). The simplest approach to this method is field inversion gel electrophoresis (FIGE).[1] FIGE works by alternating the positive and negative electrodes during electrophoresis. In this type of separation, the DNA goes periodically forward and backward. This method requires temperature control and a switching mechanism. Contour-clamped homogeneous electric field,[2] transverse alternating field electrophoresis,[3] and rotating gel electrophoresis [4,5] are examples of commonly used pulsed field or transverse angle reorientation electrophoresis. These systems require a special gel box with a special electrode and gel configuration as well as appropriate electronic control for switching the electric fields during electrophoresis. Using PFGE, the large fragments are resolved, not only by sifting through the spaces in the polymer but also by their reorientation and the time necessary to realign themselves to move in a second dimension, usually an angle of 120° (180° for FIGE) from the original direction of migration.

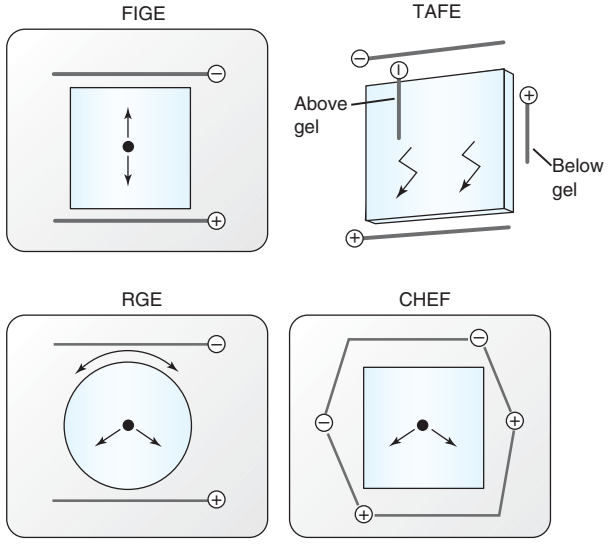


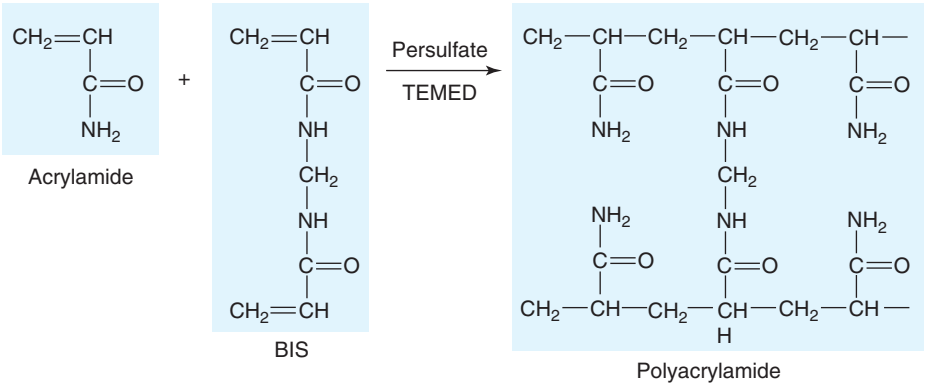
Figure 5-4 Field inversion gel electrophoresis (FIGE), contour-clamped homogeneous electric field (CHEF), transverse alternating field electrophoresis (TAFE), and rotating gel electrophoresis (RGE) are all examples of pulsed field gel configurations. Arrows indicate the migration path of the DNA.

Advanced Concepts

Field inversion gel electrophoresis (FIGE) is a special modification of PFGE in which the alternating currents are aligned 180° with respect to each other. The current pulses must be applied at different strength and/or duration so that the DNA will make net progress in one dimension. The parameters for this type of separation must be matched to the DNA being separated so that both large and small fragments of the DNA sample have time to reorient properly. For example, if timing is not sufficient for reorientation of the large fragments, small fragments will preferentially reorient and move backward and gradually lose distance with respect to the large molecules, which will continue forward progress on the next pulse cycle. Unlike PFGE that requires special equipment, FIGE can be done in a regular gel apparatus; however, its upper resolution limit is 2 megabases compared with 5 megabases for PFGE.

DNA to be resolved by these methods must be protected from breakage and shearing. Therefore, specimens are immobilized in an agarose plug before cell lysis. Further treatment of the DNA, e.g., with restriction enzymes, is also performed while the DNA is immobilized in the agarose plug. After treatment, the plug is inserted directly into the agarose gel for electrophoresis. PFGE instruments are designed to apply current in alternating directions at specific times (called the switch interval) that are set by the operator. These parameters are based on the general size of the fragments to be analyzed; i.e., a larger fragment will require a longer switch interval. PFGE is a slow migration method. Sample runs will take 24 hours or more.

Alternating field electrophoresis is used for applications that require the resolution of chromosome-sized

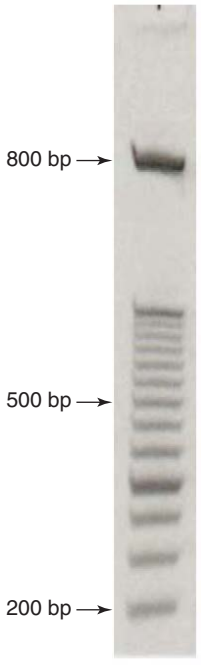


■ Figure 5-5 The repeating unit of polyacrylamide is acrylamide; bis introduces branches into the polymer.

fragments of DNA such as in bacterial typing for epidemiological purposes. Digestion of genomic DNA with restriction enzymes will yield a band pattern specific to each type of organism. By comparing band patterns, the similarity of organisms isolated from various sources can be assessed. This information is especially useful in determining the epidemiology of infectious diseases, e.g., identifying whether two biochemically identical isolates have a common source. This will be discussed in more detail in Chapter 12.

Polyacrylamide Gels

Very small DNA fragments and single-stranded DNA are best resolved on **polyacrylamide** gels in polyacrylamide gel electrophoresis (**PAGE**). Acrylamide, in combination with the cross-linker methylene bisacrylamide (Fig. 5-5), polymerizes into a gel that has consistent resolution characteristics (Fig. 5-6).



■ Figure 5-6 Resolution of double-stranded DNA fragments on a 5%, 19:1 acrylamide:bis gel.

Advanced Concepts

Different cross-linkers affect the physical nature of the acrylamide mesh. Piperazine diacrylate can reduce the background staining that may occur when the gel is stained. N,N′-bisacrylylcystamine and N,N′-diallyltartardiamide enable gels to be solubilized to enable for the easier extraction of separated products.

Polyacrylamide was originally used mostly for protein separation, but it is now routinely applied to nucleic acid analysis. Polyacrylamide gels are used for sequencing nucleic acids, mutation analyses, nuclease protection assays, and other applications requiring the resolution of nucleic acids down to the single-base level. Acrylamide is supplied to the laboratory in several forms. The powdered form is a dangerous neurotoxin and must be han-

dled with care. Solutions of mixtures of acrylamide and bis-acrylamide are less hazardous and more convenient to use. Preformed gels are the most convenient, as the procedure for preparation of acrylamide gels is more involved than that for agarose gels.

The composition of polyacrylamide gels is represented as the total percentage concentration (w/v) of monomer (acrylamide with cross-linker) T and the percentage of monomer that is cross-linker C. For example, a 6% 19:1 acrylamide:bis gel has a T value of 6% and a C value of 5%.

Unlike agarose gels that polymerize upon cooling, polymeration of polyacrylamide gels requires the use of a catalyst. The catalyst may be the nucleation agents, ammonium persulfate (APS) plus N,N,N′,N′-tetramethylethylenediamine (TEMED), or light activation. APS produces free oxygen radicals in the presence of TEMED to drive the free-radical polymerization mechanism. Free radicals can also be generated by a photochemical process using riboflavin plus TEMED. Excess oxygen inhibits the polymerization process. Therefore, **deaeration**, or the removal of air, of the gel solution is often done before the addition of the nucleation agents.

Polyacrylamide gels for nucleic acid separation are very thin, e.g., 50 μm, making gel preparation difficult. Systems have been designed to facilitate the preparation of single and multiple gels. Increasing numbers of laboratories are using preformed polyacrylamide gels to avoid the hazards of working with acrylamide and the labor time involved in gel preparation. Use of preformed gels must be scheduled, keeping in mind the limited shelf life of the product.

The main advantage of polyacrylamide over agarose is the higher resolution capability for small fragments that can be accomplished with polyacrylamide. A variation of 1 base pair in a 1-kb molecule (0.1% difference) can be detected in a polyacrylamide gel. Another advantage of polyacrylamide is that, unlike agarose, the components of polyacrylamide gels are synthetic; thus, there is not as much difference in batches obtained from different sources. Further, altering T and C in a polyacrylamide gel can change the pore size and, therefore, the sieving properties in a predictable and reproducible manner. Increasing T decreases the pore size proportionally. The minimum pore size (highest resolution for small molecules) occurs at a C value of 5%. Variation of C above or below 5% will increase pore size. Usually, C is set at 3.3% (29:1) for native and 5% (19:1) for standard DNA and RNA gels.

Capillary Electrophoresis

The widest application of **capillary electrophoresis** has been in the separation of organic chemicals such as pharmaceuticals and carbohydrates. It has also been applied to the separation of inorganic anions and metal ions. It is an alternate method to high performance liquid chromatography (HPLC) for these applications. Capillary electrophoresis has the advantage of faster analytical runs and lower cost per run than HPLC. Increasingly, capillary electrophoresis is being used for the separation and analysis of nucleic acids, which is explained below.

In this type of electrophoresis, the analyte is resolved in a thin glass (fused silica) capillary that is 30–100 cm in length and has an internal diameter of 25–100 μm. Fused silica is used as the capillary tube because it is the most transparent material allowing for the passage of fluorescent light. The fused silica is covered with a polyimide coating for protection. There is an uncoated window where the light is shone on the fragments as they pass the detector. The fused silica has a negative charge along the walls of the capillary generated by the dissociation of hydroxyl ions from the molecules of silicone. This establishes an electro-osmotic flow when a current is introduced along the length of the capillary. Under the force of the current, small and negatively charged molecules migrate faster than large and positively charged molecules (Fig. 5-7).

Capillary electrophoresis was originally applied to molecules in solution. Separation was based on their size and charge (charge/mass ratio). Optimal separation requires the use of the proper buffer to ensure that the solute is charged. Negatively charged molecules are completely ionized at high pH, whereas positively charged solutes are completely protonated in low pH buffers.

Nucleic acids do not separate well in solution. As the size or length of a nucleic acid increases (retarding migration), so does its negative charge (speeding migration), effectively confounding the charge/mass resolution. Introduction of a polymer inside the capillary restores resolution by retarding migration according to size more than charge. It is important that the nucleic acid

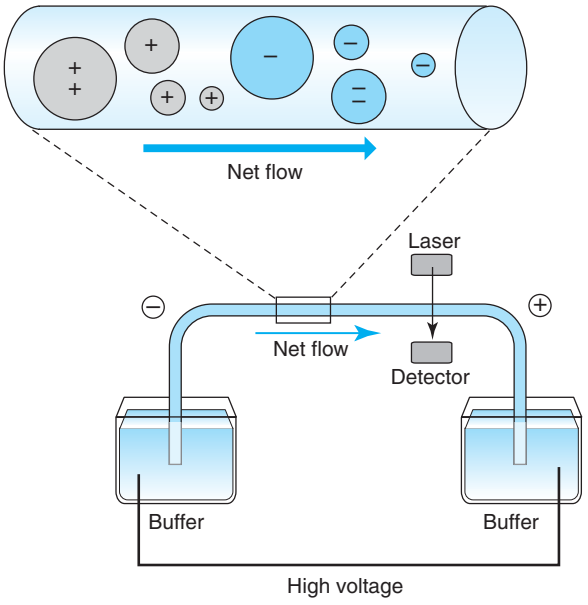


Figure 5-7 Capillary electrophoresis separates particles by size (small, fast migration; large, slow migration) and charge (negative, fast migration; positive, slow migration)

be completely denatured (single-stranded) so that it will be separated according to its size, because secondary structure will affect the migration speed. Generally, 1–50 nL of denatured nucleic acid in buffer containing formamide is introduced to the capillary, which is held at a denaturing temperature through the run.

The sample is injected into the capillary by electrokinetic, hydrostatic, or pneumatic injection. For nucleic acid analysis, electrokinetic injection is used. The platinum electrode close to the end of the capillary undergoes a transient high-positive charge to draw the sample into the end of the capillary. When the current is established, the fragments migrate through the capillary. For the resolution of nucleic acids, capillary electrophoresis is analogous to gel electrophoresis with regard to the electrophoretic parameters. The capillary's small volume, as compared with that of a slab gel, can dissipate heat more efficiently during the electrophoresis process. More efficient heat dissipation allows the technologist to run the samples at higher charge per unit area, which means that the samples migrate faster, thereby decreasing the resolution (run) time.

Nucleic acid resolution by capillary electrophoresis is used extensively in forensic applications and parentage testing performed by analyzing short tandem repeat polymorphisms. It has other applications in the clinical laboratory, such as clonality testing, microsatellite instability detection, and bone marrow engraftment analysis. Specially designed software can use differentially labeled molecular weight markers or allelic markers that, when run through the capillary with the sample, help to identify sample bands.

The capillary system has the advantages over traditional slab gel electrophoresis of increased sensitivity, so that smaller amounts of nucleic acid can be analyzed, and immediate detection of desired bands. With multiple color detection systems, standards, controls, and test samples can be run through the capillary together, thereby eliminating the lane-to-lane variations that can occur across a gel. Although instrumentation for capillary electrophoresis is costly and detection requires fluorescent labeling of samples that can also be expensive, labor and run time are greatly decreased as compared with gel electrophoresis. In addition, analytical software can automatically analyze results that are gathered by the detector in the capillary electrophoresis instrument.

Buffer Systems

The purpose of a buffer system is to carry the current and protect the samples during electrophoresis. This is accomplished through the electrochemical characteristics of the buffer components.

A buffer is a solution of a weak acid and its conjugate base. The pH of a buffered solution remains constant as the buffer molecules take up or release protons given off or absorbed by other solutes. The equilibrium between acid and base in a buffer is expressed as the dissociation constant, K_a:

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

where $[H^+]$, $[A^-]$, and $[HA]$ represent the dissociated proton, dissociated base, and associated salt concentrations, respectively. K_a is most commonly expressed as its negative logarithm, pK_a, such that

$$pK_a = -\log K_a$$

A pK_a of 2 ($K_a = 10^{-2}$) favors the release of protons. A pK_a of 12 ($K_a = 10^{-12}$) favors the association of protons.

A given buffer maintains the pH of a solution near its pKa. The amount the pH of a buffer will differ from the pKa is expressed as the Henderson-Hasselbach equation:

$$pH = pK_a + \log \frac{[\text{basic form}]}{[\text{acidic form}]}$$

If the acidic and the basic forms of the buffer in solution are of equal concentration, $pH = pK_a$. If the acidic form predominates, the pH will be less than the pK_a; if the basic form predominates, the pH will be greater than the pK_a.

The Henderson-Hasselbach equation predicts that, in order to change the pH of a buffered solution by one point, either the acidic or basic form of the buffer must be brought to a concentration of 1/10 that of the other form. Therefore, addition of acid or base will barely affect the pH of a buffered solution as long as the acidic or basic forms of the buffer are not depleted.

Control of the pH of a gel by the buffer also protects the sample molecules from damage. Furthermore, the current through the gel is carried by buffer ions, preventing severe fluctuations in the pH of the gel.

A buffer concentration must be high enough to provide sufficient acidic and basic forms to buffer its solution. Raising the buffer concentration, however, also increases the conductivity of the electrophoresis system, generating more heat at a given voltage. This can cause problems with gel stability and can increase sample denaturation. High buffer concentrations must therefore be offset by low voltage.

In order for nucleic acids to migrate properly, the gel system must be immersed in a buffer that conducts the electric current efficiently in relation to the buffering capacity. Ions with high-charge differences, +2, -2, +3, etc., move through the gel more quickly; that is, they increase conductivity without increasing buffering capacity. This results in too much current passing through the gel as well as faster depletion of the buffer. Therefore, buffer components such as Tris base or borate are preferred because they remain partly uncharged at the desired pH and thus maintain constant pH without high conductivity.

In addition to pKa, charge, and size, other buffer characteristics that can be taken into account when choosing a buffer include toxicity, interaction with other components, solubility, and ultraviolet absorption.

The Tris buffers Tris borate EDTA (TBE; 0.089 M Tris-base, 0.089 M boric acid, 0.0020 M EDTA), Tris phosphate EDTA (TPE; 0.089 M Tris-base, 1.3% phosphoric acid, 0.0020 M EDTA) and Tris acetate EDTA (TAE; 0.04 M Tris-base, 0.005 M sodium acetate, 0.002 M EDTA) are most commonly used for electrophoresis of DNA. There are some advantages and disadvantages of both TBE and TAE that must be considered before one of these buffers is used for a particular application. TBE has a greater buffering capacity than TAE. Although the ion species in TAE are more easily exhausted during extended or high-voltage electrophoresis, DNA will migrate twice as fast in TAE than in TBE in a constant current. TBE is not recommended for some post-electrophoretic isolation procedures. When using any buffer, especially TBE and TPE, care must be taken that the gel does not overheat when run at high voltage in a closed container. Finally, stock solutions of TBE are prone to precipitation. This can result in differences in concentration between the buffer in the gel and the running buffer. Such a gradient will cause localized distortions in nucleic acid migration

Advanced Concepts

The Henderson-Hasselbach equation also predicts the concentration of the acidic or basic forms at a given pH. It can be used to calculate the state of ionization, i.e., the predominance of acidic or basic forms, of a species in solution. A buffer should be chosen that has a pK_a within a half point of the desired pH, which is about 8.0 for nucleic acids.

Advanced Concepts

The migration of buffer ions is not restricted by the gel matrix, so the speed of their movement under a current is governed strictly by the size of the ion and its charge (charge/mass ratio). Tris is a relatively large molecule, so its charge-to-mass ratio is low, and it moves through the current relatively slowly, even at high concentrations, giving increased buffering capacity.

patterns, often causing a salt wave that is visible as a sharp horizontal band through the gel.

Buffer Additives

Buffer additives are often used to modify sample molecules in ways that affect their migration. Examples of these additives are formamide, urea, and various detergents. **Denaturing agents**, such as formamide or urea, break hydrogen bonds between complementary strands or within the same strand of DNA or RNA. The conformation or solubility of molecules can be standardized by the addition of one or both of these agents. Formamide and heat added to DNA and RNA break and block the hydrogen bonding sites, hindering complementary sequences from reannealing. As a result, the molecules become long, straight, unpaired chains. Urea and heat in the gel systems maintain this conformation such that intrachain hybridization (folding) of the nucleic acid molecules does not affect migration speeds, and separation can occur strictly according to the size or length of the molecule.

Electrophoresis of RNA requires different conditions imparted by different additives than are used with DNA. Because RNA is single-stranded and it tends to fold to optimize internal homology, it must be completely denatured to prevent folding in order to accurately determine its size by migration in a gel system. The secondary structures formed in RNA are strong and more difficult to denature than DNA homologies. Denaturants used for RNA include methylmercuric hydroxide (MMH), which reacts with amino groups on the RNA preventing base pairing between homologous nucleotides, and aldehydes (e.g., formaldehyde, glyoxal), which also disrupt base pairing. MMH is not used routinely because of its extreme toxicity.

RNA can be separated in 10-mM sodium phosphate, pH 7, or MOPS buffer (20 mM 3-[N-morpholino] propanesulfonic acid, pH 7, 8-mM sodium acetate, 1 mM EDTA, pH 8). The RNA sample is incubated in dimethyl sulfoxide, 1.1 M glyoxal (ethane 1.2 dione) and 0.01 M sodium phosphate, pH 7, to denature the RNA prior to loading the sample on the gel. Due to **pH drift** (decrease of pH at the cathode [-] and increase of the pH at the anode [+]) during the run, the buffer should be recirculated from the anode end of the bath to the cathode end (Fig. 5-8). This can be accomplished using a peristaltic pump or by stopping the gel at intervals and transferring the buffer from the cathode to the anode ends.

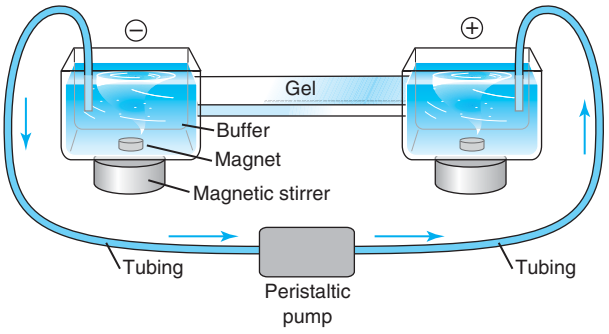


Figure 5-8 A peristaltic pump can be used to recirculate buffer from the cathode to the anode end while running a denaturing gel.

Electrophoresis Equipment

Gel electrophoresis can be done in one of two conformations, horizontal or vertical. In general, agarose gels are run horizontally, and polyacrylamide gels are run vertically.

Horizontal gels are run in acrylic containers called gel boxes or baths that are divided into two parts with a platform in the middle on which the gel rests (Fig. 5-9). Platinum wires make up the electrodes in the gel compartments. The wires are connected to a power source by banana clips or connectors through the walls of the container. The gel in the box is submerged with electrophoresis buffer filling both compartments and making a continuous system through which the current flows. The thickness of the gel and the volume of the buffer affect migration, so these parameters should be kept constant for consistent results. As the gel is submerged through the loading and electrophoresis process, horizontal gels are sometimes referred to as **submarine gels**. The power supply will deliver voltage, setting up a current that will run through the gel buffer and the gel, carrying the charged sample through the matrix of the gel at a speed corresponding to the charge/mass ratio of the sample molecules.

Horizontal agarose gels are cast as square or rectangular slabs of varying size. Purchased gel boxes come with casting trays that mold the gel to the appropriate size for the gel box. The volume of the gel solution will deter-

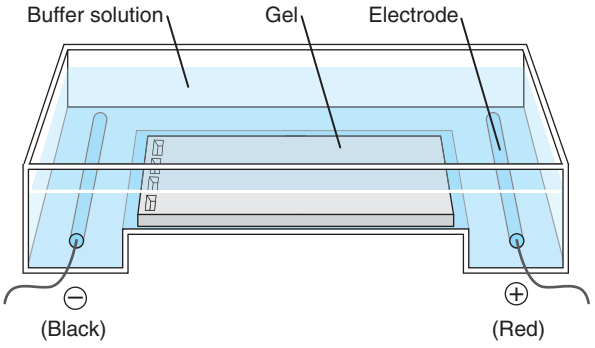


Figure 5-9 A typical horizontal submarine gel system. A red connector is attached to the positive outlet on the power supply and a black to the negative port.

mine the thickness of the gel. Agarose, supplied as a dry powder, is mixed at a certain percentage (w/v) with electrophoresis buffer and heated on a heat block or by microwave to dissolve and melt the agarose. The molten agarose is cooled to 55°–65°C, and a certain volume is poured into the casting tray as dictated by the gel box manufacturer or application. A comb is then inserted into the top of the gel to create holes, or **wells**, in the gel into which the sample will be loaded. The size of the teeth in the comb will determine the volume of loaded sample and the number of teeth will determine the number of wells that are available in the gel to receive samples. The gel is then allowed to cool, during which time it will solidify. After the gel has polymerized, the comb is carefully removed and the gel is placed into the gel box and submerged in electrophoresis buffer.

Advanced Concepts

Self-contained agarose gel systems have been developed to facilitate the electrophoresis process. They are manufactured in closed plastic cassettes containing buffer, gel, and stain. These are convenient for routine use, but restrict the gel configuration, i.e., number and size of wells, etc. Also, the percentage of agarose or acrylamide is limited to what is available from the manufacturer. Furthermore, the separated nucleic acids can not be removed from these closed cassettes, limiting their analysis.

Vertical gel boxes have separate chambers that are connected by the gel itself. Electrodes are attached to the upper and lower buffer chambers to set up the current that will run through the gel. The gel must be in place before filling the upper chamber with buffer. Some systems have a metal plate attached to the back of the gel to maintain constant temperature across the gel. Maintaining constant temperature throughout the gel is more of a problem with vertical gels because the outer edges of the gel cool more than the center, slowing migration in the outer lanes compared with lanes in the center of the gel. This is called "gel smiling" because similar-sized bands in the cooler outer lanes will migrate slower than comparable bands in the inside lanes. Ensuring that there is no variation in temperature across the gel prevents gel smiling from occurring.

Vertical gel systems can range from large sequencing systems (35 cm × 26 cm) to mini-systems (8 cm × 10 cm). Some mini-systems are big enough to accommodate two gels at a time (Fig. 5-10). Mini-systems are used extensively for analyses that do not require single base

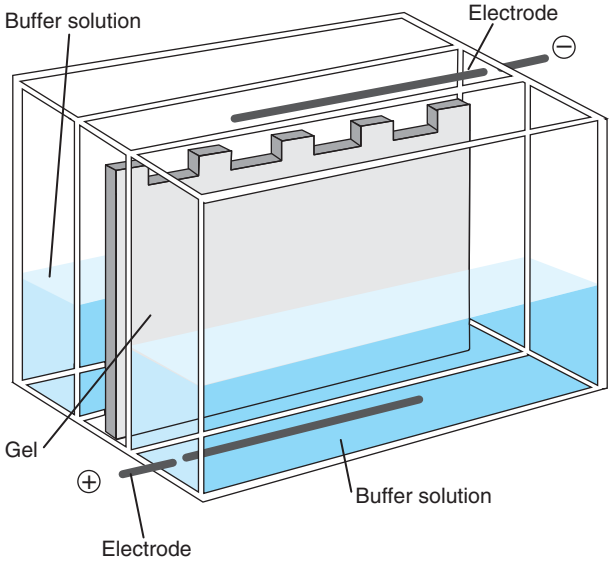


Figure 5-10 A typical vertical gel apparatus. Polymerized gels are clamped into the gel insert (left) and placed in the gel bath (right). The positive electrode will be in contact with the bottom of the gel and the buffer, filling about a third of the gel bath. The negative electrode will be in contact with the top of the gel and a separate buffer compartment in the top of the insert.

pair resolution. The larger systems are used for sequencing or other procedures requiring single-base resolution. The gels are loaded from the top, below a layer of buffer in the upper chamber. Long, narrow gel-loading pipette tips that deposit the sample neatly on the floor of the well increase band resolution and sample recovery.

Vertical gels are cast between glass plates that are separated by spacers. The spacers determine the thickness of the gel, ranging 0.05–4 mm. The bottom of the gel is secured by tape or by a gasket in specially designed gel casting trays. After addition of polymerization agents, the liquid acrylamide is poured or forced between the glass plates with a pipet or a syringe. The comb is then placed on the top of the gel. During this process, it is important not to introduce air into the gel or beneath the comb. Bubbles will form discontinuities in the gel, and oxygen will inhibit the polymerization of the acrylamide. The comb is of a thickness equal to that of the spacers so that the gel will be the same thickness throughout. As with horizontal gels, the number and size of the comb teeth determine the number of wells in the gel and the sample volume that can be added to each well. Specialized combs, called **shark's-tooth combs**, are often used for sequencing gels (Fig. 5-11). These combs are placed upside down (teeth up, not in contact with the gel) to form a trough on the gel during polymerization. After polymerization is complete, the comb is removed and placed tooth-side down on top of the gel for loading. With this configuration, the spaces *between* the comb teeth form the wells as opposed to the teeth themselves forming the wells in the horizontal gels. The advantage to this arrangement is that the lanes are placed immediately adjacent to one another to facilitate lane-to-lane comparisons. When used, standard combs are removed before the gel is loaded, whereas the shark's-tooth combs are made so that the wells can be loaded while the comb is in place. When the standard combs are removed from the gel, care must be taken not to break or displace the "ears" that were formed by the spaces between the teeth in the comb that separate the gel wells.

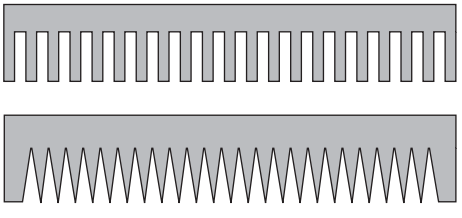


Figure 5-11 Combs for polyacrylamide electrophoresis. Regular combs (top) have teeth that form the wells in the gel. Shark's-tooth combs (bottom) are placed onto the polymerized gel, and the sample is loaded between the teeth of the comb.

Polyacrylamide gels can also be cast in tubes for isoelectric focusing or two-dimensional gel electrophoresis. The tubes containing the gels are placed into a chamber separated as for vertical slab gels. The tubes are held in place by gaskets in the upper chamber. This gel configuration, however, limits the number of samples, as only one sample can be run per gel.

Gel Loading

Prior to loading the sample containing isolated nucleic acid onto the gel, **tracking dye** and a density agent are added to the sample. The density agent (either Ficoll, sucrose, or glycerol) increases the density of the solution as compared with the electrophoresis buffer. When the sample solution is dispensed into the wells of the gel below the surface of the buffer, it sinks into the well instead of floating away in the buffer. The tracking dyes are used to monitor the progress of the electrophoresis run. The dyes migrate at specific speeds in a given gel concentration and usually run ahead of the smallest fragments of DNA (compare Table 5.1, 5.2, and 5.3). They are not associated with the sample DNA, and thus they do not affect the separation of the sample DNA. The movement of the tracking dye is monitored, and when the tracking dye approaches the end of the well electrophoresis is terminated. Bromophenol blue is a tracking dye that is used for many applications. Xylene cyanol green is another example of chromophores that are used as tracking dyes for both agarose and polyacrylamide gels.

Advanced Concepts

A type of "bufferless" electrophoresis system supplies buffer in gel form or strips. These are laid next to the preformed gel on a platform that replaces the electrophoresis chamber. These systems can offer the additional advantage of precise temperature control during the run.

Advanced Concepts

Gels in cassette systems and gel strip systems can be loaded without loading buffer because the wells are "dry," precluding the need for density gradients. As these systems also have automatic shut-off at the end of the run, tracking dye is usually not necessary, although some of these systems have a tracking dye built into the gel and/or buffer.

Detection Systems

Following are the status of samples during and after electrophoresis is accomplished using dyes that specifically associate with nucleic acid. The agents used most frequently for this application are fluorescent dyes and silver stain.

Nucleic Acid–Specific Dyes

Intercalating agents intercalate, or stack, between the nitrogen bases in double-stranded nucleic acid. Ethidium bromide, 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (EtBr), is one of these agents. Under excitation with ultraviolet light at 300 nm, EtBr in DNA emits visible light at 590 nm. Therefore, DNA separated in agarose or acrylamide and exposed to EtBR will emit orange light when illuminated by ultraviolet light at 300 nm. EtBr was the most widely used dye in early DNA and RNA analyses. Care must be taken in handling EtBr because it is carcinogenic. After electrophoresis, the agarose or acrylamide gel can be soaked in a solution of 0.1–1–mg/ml EtBr in running buffer (TAE, TBE, or TPE) or TE. Alternatively, dye can be added directly to the gel before polymerization or to the running buffer. The latter two measures save time and allow visualization of the DNA during the run. Dye added to the gel, however, may form a bright front across the gel that could mask informative bands. Dye added to the running buffer produces more consistent staining, although more hazardous waste is generated by this method. Some enclosed gel systems contain EtBr inside a plastic enclosed gel cassette, limiting exposure and waste. After soaking or running in EtBr, the DNA illuminated with ultraviolet light will appear as orange bands in the gel. The image can be captured with a camera or by digital transfer to analytical software.

Table 5.3 **Tracking Dye Comigration***

Gel %	Bromophenol Blue (Nucleotides)	Xylene Cyanol (Nucleotides)
Agarose		
0.5–1.5	300–500	4000–5000
2.0–3.0	80–120	700–800
4.0–5.0	20–30	100–200
PAGE		
4	95	450
6	60	240
8	45	160
10	35	120
12	20	70
20	12	45

*Migration depends on buffer type (TAE, TBE, or TPE) and the formulation of agarose, acylamide, and bis.

SyBr green is one of a set of stains introduced in 1995 as another type of nucleic acid–specific dye system. It differs from EtBr in that it does not intercalate between bases; it sits in the minor groove of the double helix. SyBr green in association with DNA or RNA also emits light in the orange range. SyBr green staining is 25–100 times more sensitive than EtBr (detection level: 60 pg of double-stranded DNA vs. 5 ng for EtBr). This is due, in part, to background fluorescence from EtBr in agarose. A 1× dilution of the manufacturer's 10,000X stock solution of SyBr green in TAE, TBE, or TE can be used in methods described for EtBr. A 1/100 dilution of SyBr green can also be added directly to the DNA sample before electrophoresis. DNA prestaining decreases the amount of dye required for DNA visualization but lowers the sensitivity of detection and may, at higher DNA concentrations, interfere with DNA migration through the gel.[6] Because SyBr green is not an intercalating agent, it is not as mutagenic.[7]

Although SyBr green has some advantages over EtBr, many laboratories continue to use the latter dye due to the requirement for special optical filters for detection of SyBr green. Scanning and photographic equipment optimized for EtBr would have to be modified for optimal detection of the SyBr green stains. New instrumentation with more flexible detection systems allows utilization of

the SyBr green stains. SyBr green is the preferred dye for real-time PCR methods.

Silver Stain

A more sensitive staining system originally developed for protein visualization is silver stain. After electrophoresis, the sample is fixed with methanol and acetic acid. The gel is then impregnated with ammoniacal silver (silver diamine) solutions or silver nitrate in a weakly acid solution.[8] Interaction of silver ions with acidic or nucleophilic groups on the target results in crystallization or deposition of metallic silver under optimal pH conditions. The insoluble black silver salt precipitates upon introduction of formaldehyde in a weak acid solution or alkaline solution for sliver nitrate. Of the two procedures, silver diamine is best for thick gels, whereas silver nitrate is considered to be more stable.[9]

Silver staining avoids the hazards of the intercalators, but silver nitrate is itself also a biohazard. In addition, silver staining is more complicated than simple intercalation. Color development must be carefully watched as the precipitate accumulates in order to stop the reaction once optimal signal is reached. Overexposure of the gel will result in high backgrounds and masking of results. The increased sensitivity of this staining procedure, however, makes up for its limitations. It is especially useful for protein analysis and for detection of limiting amounts of product.

• STUDY QUESTIONS •

1. You wish to perform a resolution of your restriction enzyme–digested DNA fragments. The size of the expected products ranges 500–100 bp. You discover two agarose gels polymerizing on the bench. One is 5% agarose; the other is 2% agarose. Which one might you use to resolve your fragments?

2. After completion of the run of fragments along with the proper molecular weight standard on the agarose gel, suppose a. or b. below was observed. What might be explanations for these? (Assume you have included a molecular weight marker in your run.)
   a. The gel is blank (no bands, no molecular weight standard).
   b. Only the molecular weight standard is visible.

3. How does PFGE separate larger fragments more efficiently than standard electrophoresis?

4. A 6% solution of 19:1 acylamide is mixed, deaerated, and poured between glass plates for gel formation. After an hour, the solution is still liquid. What might be one explanation for the gel not polymerizing?

5. A gel separation of RNA yields aberrantly migrating bands and smears. Suggest two possible explanations for this observation?

6. Why does DNA not resolve well in solution (without a gel matrix)?

7. Why is SyBr green less toxic than EtBr?

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Chapter 6 *Lela Buckingham*

Analysis and Characterization of Nucleic Acids and Proteins

OUTLINE

OBJECTIVES

- Describe how restriction enzyme sites are mapped on DNA.
- Construct a restriction enzyme map of a DNA plasmid or fragment.
- Diagram the Southern blot procedure.
- Explain depurination and denaturation of resolved DNA.
- Describe the procedure involved in blotting (transfer) DNA from a gel to a membrane.
- Discuss the purpose and structure of probes that are used for blotting procedures.
- Define hybridization, stringency, and melting temperature.
- Calculate the melting temperature of a given sequence of dsDNA.
- Compare and contrast radioactive and nonradioactive DNA detection methods.
- Compare and contrast dot and slot blotting methods.
- Describe microarray methodology.
- Discuss solution hybridization.

Restriction Enzyme Mapping

Clinical and forensic analyses require characterization of specific genes or genomic regions at the molecular level. Because of their sequence-specific activity (see Chapter 1), **restriction endonucleases** provide a convenient tool for molecular characterization of DNA.

Restriction enzymes commonly used in the laboratory have four to six base pair **recognition sites**, or binding/cutting sites, on the DNA. Any four to six base pair nucleotide sequence occurs at random in a sufficiently long stretch of DNA. Therefore, restriction sites will occur naturally in DNA. Restriction site mapping, i.e., determining where in the DNA sequence a particular restriction enzyme recognition site is located, was initially developed using small circular bacterial plasmids. The resultant maps were used to identify and characterize naturally occurring plasmids and to engineer the construction of recombinant plasmids.

To make a **restriction map**, DNA is exposed to several restriction enzymes separately and then in particular combinations. Take, for example, a linear fragment of DNA cut with the enzyme *Pst*I. After incubation with the enzyme, the resulting fragments are separated by gel electrophoresis. The gel image reveals four fragments, labeled A, B, C, and D, produced by *Pst*I (Fig. 6-1). From the number of fragments one can deduce the number of *Pst*I sites: three. The sizes of the fragments, as determined by comparison with known molecular-weight standards, indicate the distance between these sites or from the site to the end of the fragment. Although *Pst*I analysis of this fragment yields a characteristic four-band restriction pattern, it does not indicate the order of the four restriction products in the original fragment. To begin to determine

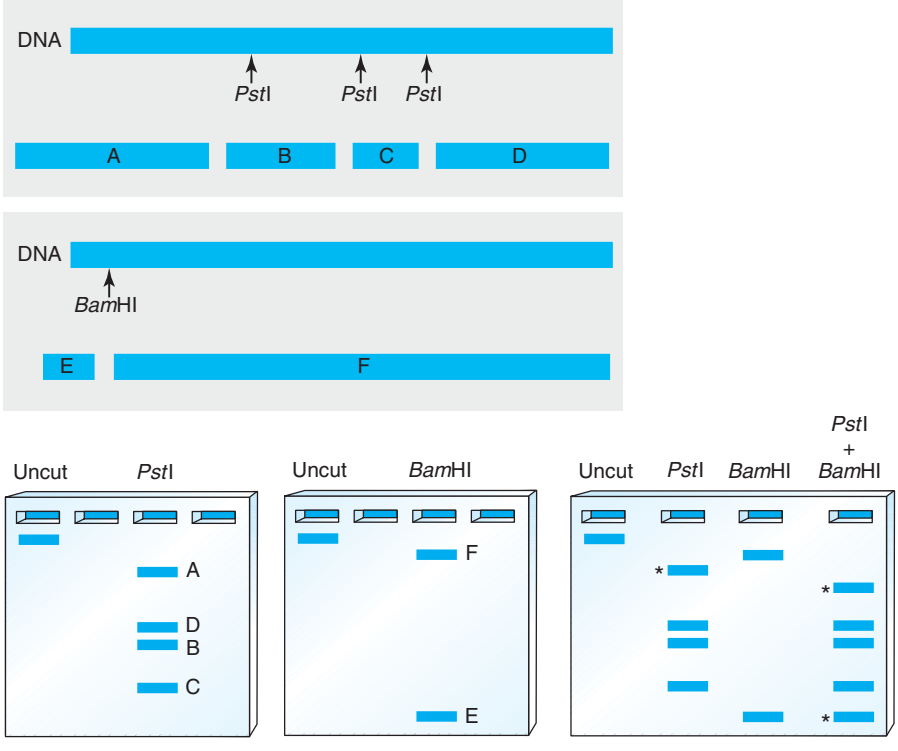


Figure 6-1 Restriction mapping of a linear DNA fragment (top green bar). The fragment is first cut with the enzyme *Pst*I. Four fragments result as determined by agarose gel electrophoresis indicating that there are three *Pst*I sites in the linear fragment. The size of the pieces indicates the distance between the restriction sites. A second cut with *Bam*HI (bottom) yields two fragments, indicating one site. Since one *Bam*HI fragment (E) is very small, the *Bam*HI site must be near one end of the fragment. Cutting with both enzymes indicates that the *Bam*HI site is in the *Pst*I fragment A.

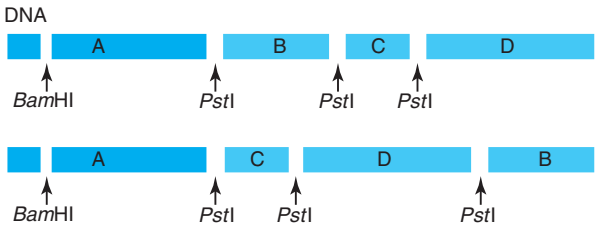


Figure 6-2 Two possible maps inferred from the observations described in Figure 6-1. The *Bam*HI site positions fragment A at one end (or the other) of the map. Determination of the correct map requires information from additional enzyme cuts.

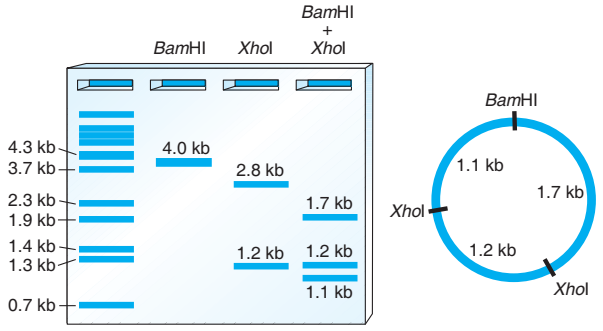


Figure 6-3 Restriction mapping of a plasmid. After incubating plasmid DNA with restriction enzymes, agarose gel electrophoresis banding patterns indicate the number of restriction sites and the distance between them.

the order of the restriction fragments, another enzyme is used, for example *Bam*HI. Cutting the same fragment with *Bam*HI yields two pieces, indicating one *Bam*HI site in this linear fragment (see Fig. 6-1). Observe that one restriction product (F) is very much larger than the other (E). This means that the *Bam*HI site is close to one end of the fragment. When the fragment is cut simultaneously with *Pst*I and *Bam*HI, five products are produced, with *Pst*I product A cut into two pieces by *Bam*HI. This indicates that A is on one end of the DNA fragment. By measuring the number and length of products produced by other enzymes, the restriction sites can be placed in linear order along the DNA sequence. Figure 6-2 shows two possible maps based on the results of cutting the fragment with *Pst*I and *Bam*HI. With adequate enzymes and enzyme combinations, a detailed map of this fragment can be generated.

Mapping of a circular plasmid is slightly different, as there are no free ends (Fig. 6-3). The example shown in the figure is a 4-kb pair circular plasmid with one *Bam*HI site and two *Xho*I sites. Cutting the plasmid with *Bam*HI will yield one fragment. The size of the fragment is the size of the plasmid. Two fragments released by *Xho*I indicate that there are two *Xho*I sites in the plasmid and that these sites are 1.2 and 2.8 kb pairs away from each other. As with linear mapping, cutting the plasmid with *Xho*I and *Bam*HI at the same time will start to order the sites with respect to one another on the plasmid. One possible arrangement is shown in Figure 6-3. As more enzymes are used, the map becomes more detailed.

The pattern of fragments produced by restriction enzyme digestion can be used to identify that DNA and to monitor certain changes in the size, structure, or sequence of the DNA. Because of inherited or somatic differences in the nucleotide sequences in human DNA, the number or location of restriction sites for a given restriction enzyme are not all the same in all individuals. The location and order of restriction enzyme sites on a DNA fragment is a molecular characteristic of that DNA. The resulting differences in the size or number of restriction fragments are called **restriction fragment length polymorphisms** (RFLPs). RFLPs were the basis of the first molecular-based human identification and mapping methods. RFLPs can also be used for the clinical analysis of structural changes in chromosomes associated with disease (translocations, deletions, insertions, etc.).

Hybridization Technologies

Procedures performed in the clinical molecular laboratory are aimed at specific targets in genomic DNA. This requires visualization or detection of a specific gene or region of DNA in the background of all other genes. There are several ways to find a particular region of DNA from within an isolated DNA sample. The initial method for molecular analysis of specific DNA sites within a complex background was the Southern blot. Modifications of the Southern blot are applied to analysis of RNA and protein in order to study gene expression and regulation (Table 6.1).

Southern Blots

The **Southern blot** is named for Edwin Southern, who first reported the procedure.[1] In the Southern blot, DNA

Table 6.1 **Hybridization Technologies**

Hybridization Method	Target	Probe	Purpose
Southern blot	DNA	Nucleic acid	Gene structure
Northern blot	RNA	Nucleic acid	Transcript structure, processing, gene expression
Western blot	Protein	Protein	Protein processing, gene expression
Southwestern blot	Protein	DNA	DNA binding proteins, gene regulation

is isolated and cut with restriction enzymes. The fragments are separated by gel electrophoresis, depurinated and denatured, and then transferred to a solid support such as nitrocellulose. In the final steps of the procedure, the DNA fragments are exposed to a labeled **probe** (complementary DNA or RNA) that is specific in sequence to the region of interest, unbound probe is removed, and the signal of the probe is detected to indicate the presence or absence (lack of signal) of the sequence in question. The original method entailed hybridization of a radioactively-labeled probe to detect the DNA region to be analyzed. As long as there is a probe of known identity, this procedure can analyze any gene or gene region in the genome at the molecular level. The following sections will describe the parts of the Southern blot procedure in detail as well as discuss modifications of the procedure in order to analyze RNA and protein.

Restriction Enzyme Cutting and Resolution

After DNA isolation, the first step in the Southern blot procedure is digestion of the DNA with restriction enzymes. The choice of enzymes used will depend on the applications. For routine laboratory tests, restriction maps of the target DNA regions will have previously been determined, and the appropriate enzymes will be recommended. For other methods, such as typing of unknown organisms or cloning, several enzymes may be tested to find those that will be most informative.

Ten to 50 μg of genomic DNA are used for each restriction enzyme digestion for Southern analysis. More or less DNA may be used depending on the sensitivity of the detection system, the volume and configurations of wells, and the abundance of the target DNA. In the clinical laboratory, specimen availability may limit the amount of DNA that can be used.

After restriction enzyme digestion, the resulting fragments are resolved by gel electrophoresis. The percentage and nature of the gel will depend on the size of the DNA region to be analyzed (see Chapter 5, Tables 5.1 and 5.2). As with all electrophoresis, a molecular weight standard should be run with the test samples. After electrophoresis, it is important to observe the cut DNA. Figure 6-4a shows a gel stained with ethidium bromide and illuminated by ultraviolet (UV) light. Genomic DNA cut with restriction enzymes should produce a smear representing the billions of fragments of all sizes released by the enzyme cutting. The brightness of the DNA smears should be similar from lane to lane, assuring that equal amounts of DNA were added to all lanes. In any lane, a large aggregate of DNA near the top of the gel indicates that the restriction enzyme activity was incomplete. A smear located primarily in the lower region of the lane is a sign that the isolated DNA is degraded. Either of these two latter conditions will prevent accurate analysis. If either is observed, the DNA isolation and/or the restriction digest should be repeated accordingly.

Preparation of Resolved DNA for Blotting (Transfer)

The goal of the Southern blot procedure is to analyze a specific region of the sample DNA. First, the DNA sample is digested, i.e., cut using a variety of restriction endonucleases, and then the DNA fragments are separated by electrophoresis. The resultant restriction fragments containing the target sequence to be analyzed are obviously not distinguishable in the smear from other fragments that do not have the target sequence. Target fragments can be detected by hybridization with a homologous sequence of single-stranded DNA or RNA labeled with a detectable marker. To achieve optimal hydrogen bonding between the probe and its complementary sequence in the resolved sample DNA, the double-stranded DNA fragments in the gel must be denatured and transferred to a nitrocellulose membrane.

Depurination

Before moving the DNA fragments from the gel to the membrane for blotting, the double-stranded DNA fragments must be **denatured**, or separated, into single strands. This is performed as the DNA remains in place

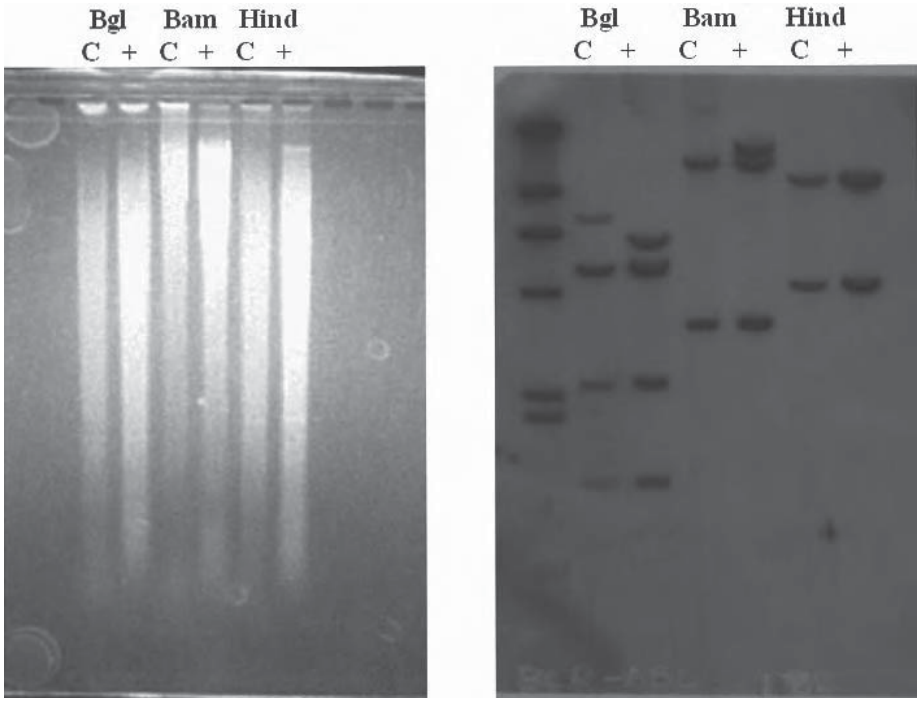


Figure 6-4 (a.) Genomic DNA fragments cut with restriction enzymes *Bgl* II, *Bam*HI and *Hind* III and separated by gel electrophoresis. (b.) Autoradiogram of the fragments hybridized to a radioactive or chemiluminescent probe. Control lanes, C, show the restriction pattern of normal DNA. Test lanes, +, show the different restriction patterns that result from the abnormal or translocated DNA.

in the gel. Although short fragments can be denatured directly as described below, larger fragments (>500 bp) are more efficiently denatured if they are **depurinated** before denaturation (Fig. 6-5). Therefore, for large fragments, the gel is first soaked in HCl solution, a process that removes purine bases from the sugar phosphate backbone. This will "loosen up" the larger fragments for more complete denaturation.

Denaturation

Following depurination, the DNA is denatured by exposing the DNA in the gel to sodium hydroxide. The strong base (NaOH) promotes breakage of the hydrogen bonds holding the DNA strands to one another. The resulting single strands are then available to hydrogen-bond with the single-stranded probe. Further, the single-stranded DNA will bind more tightly than double-stranded DNA to the nitrocellulose membrane upon transfer.

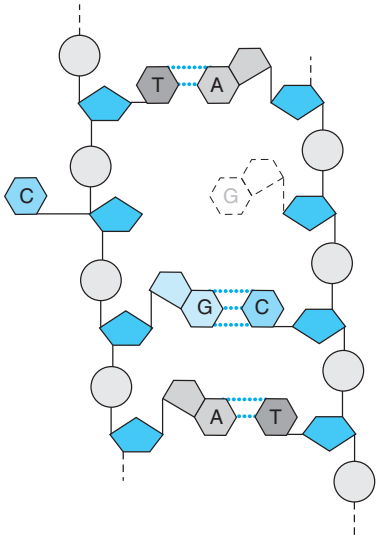


Figure 6-5 An apurinic site in double-stranded DNA. Loss of the guanine (right) leaves an open site but does not break the sugar phosphate backbone of the DNA.

Advanced Concepts

Treatment of DNA with dilute (0.1–0.25 mM) hydrochloric acid results in hydrolysis of the glycosidic bonds between purine bases and the sugar of the nucleotides. This loss of purines (adenines and guanines) from the sugar-phosphate backbone of DNA leaves apurinic sites (Fig. 6-5). The DNA backbone remains intact and holds the rest of the bases in linear order. Removal of some of the purine bases promotes the subsequent breaking of hydrogen bonds between the two strands of the DNA during the denaturation step in Southern blotting.

Advanced Concepts

Binding of single-stranded DNA to nitrocellulose does not prevent hydrogen-bond formation of the immobilized DNA with complementary sequences. Although not covalent, the bond between the membrane and the DNA is much stronger than the hydrogen bonds that hold complementary strands together. This allows removal of probes and reprobing of immobilized fragments if necessary.

Blotting (Transfer)

Before exposing the denatured sample DNA to the probe, the DNA must be **transferred**, or blotted, to a solid substrate that will facilitate probe binding and signal detection. This substrate is usually nitrocellulose, nitrocellulose on an inert support, nylon or cellulose modified with a diethyl amino ethyl, or a carboxy methyl (CM) chemical group. Membranes of another type, polyvinyl difluoride (PVDF), are used for immobilizing proteins for probing with antibodies (**Western blots**).

Membrane Types

Single-stranded DNA avidly binds to nitrocellulose membranes with a noncovalent, but irreversible, connection. The binding interaction is hydrophobic and electrostatic between the negatively charged DNA and the positive charges on the membrane. Nitrocellulose-based membranes bind 70–150 μg of nucleic acid per square centimeter. Membrane pore sizes (0.05 microns to 0.45 microns) are suitable for small DNA fragments up to fragments >20,000 bp in length.

Pure nitrocellulose has a high binding capacity for proteins as well as nucleic acids. It is the most versatile medium for molecular transfer applications. It is also compatible with different transfer buffers and detection systems. Nitrocellulose is not as sturdy as other media and becomes brittle with multiple reuses. **Reinforced nitrocellulose** is more appropriate for applications where multiple probings may be necessary. Mechanically stable membranes can be formulated with a net neutral charge to decrease the background. These membranes have a very high binding capacity (<400 $\mu g/cm^2$), which increases sensitivity. A covalent attachment of nucleic acid to these membranes is achieved by UV cross-linking.

Membranes with a positive charge more effectively bind small fragments of DNA. These membranes, however, are more likely to retain protein or other contaminants that will contribute to background after the membrane is probed.

Before transfer of the sample, membranes are moistened by floating them on the surface of the transfer buffer. Any dry spots (areas where the membrane does not properly hydrate) will remain white while the rest of the membrane darkens with buffer. If the membrane does not hydrate evenly, dry spots will inhibit binding of the sample.

Transfer Methods

Transfer can be performed in several ways. The goal of all methods is to move the DNA from the gel to a membrane substrate for probing.

Advanced Concepts

Diethylaminoethyl (DEAE)-conjugated cellulose effectively binds nucleic acids and negatively charged proteins. Polyvinylidene difluoride (**PVDF**) and charged carboxy methyl cellulose membranes are used only for protein (Western) blotting. These membranes bind nucleic acid and proteins by hydrophobic and ionic interactions with a binding capacity of 20–40 $\mu g/cm^2$ to 150 $\mu g/cm^2$ for PVDF.

Advanced Concepts

Capillary transfer is simple and relatively inexpensive, as no instruments are required. The transfer, however, can be less than optimal, especially with large gels. Bubbles or crystals in between the membrane and the gel can cause loss of information or staining artifacts. The procedure is also slow, taking from a few hours to overnight for large fragments.

The original method developed by Southern used **capillary transfer** (Fig. 6-6). For capillary transfer, the gel is placed on top of a reservoir of buffer, which can be a shallow container or membrane papers soaked in high salt buffer, e.g., 10X saline sodium citrate (10X SSC: 1.5 M NaCl, 0.15 M Na citrate) or commercially available transfer buffers. The nitrocellulose membrane is placed directly on the gel, and dry absorbent membranes or paper towels are stacked on top of the membrane. The buffer is moved by capillary action from the lower reservoir to the dry material on top of the gel. The movement of the buffer through the gel will carry the denatured DNA out of the gel. When the DNA contacts the nitrocellulose membrane, the DNA will bind to it while the buffer will pass through to the membranes or paper towels on top.

A second method, called **electrophoretic transfer**, uses electric current to move the DNA from the gel to the membrane (Fig. 6-7). This system utilizes electrodes attached to membranes above (anode) and below (cathode) the gel. The current carries the DNA transversely from the gel to the membrane. Electrophoretic transfer is carried out with a "tank" or by a "semidry" approach. In the tank method, the electrodes transfer current through the membrane through electrophoresis buffer as shown in the figure. In the semidry method, the electrodes contact the gel-membrane sandwich directly, requiring only enough buffer to soak the gel and membrane. The tank electrophoretic transfer is preferred for large proteins resolved on acrylamide gels, whereas the semidry method is frequently used for small proteins.

Vacuum transfer is a third method of DNA blotting (Fig. 6-8). This blotting technique uses suction to move the DNA from the gel to the membrane in a recirculating buffer. Like electrophoretic transfer, this method transfers the DNA more rapidly, e.g., in hours rather than days, than capillary transfer. Also, discontinuous transfer due to air trapped between the membrane and the gel is avoided. One disadvantage of the second and third methods is the expense and maintenance of the electrophoresis and vacuum equipment.

After binding the nucleic acid to membranes, the cut, denatured DNA is permanently immobilized to the membrane by baking the membrane in a vacuum oven (80°C, 30–60 min.) or by UV cross-linking, i.e., covalently attaching the DNA to the nitrocellulose using UV light energy. The purpose of baking or cross-linking is to prevent the transferred DNA fragments from washing away or moving on the membrane.

Following immobilization of the DNA, a **prehybridization** step is required to prevent the probe from binding to nonspecific sites on the membrane surface, which will cause high background. Prehybridization involves incubating the membrane in the same buffer in which the probe will subsequently be introduced. At this point, the buffer does not contain probe. The buffer consists of blocking agents such as Denhardt solution (Ficoll, polyvinyl pyrrolidane, bovine serum albumin) and salmon sperm DNA. Sodium dodecyl sulfate (SDS, 0.01%) may also be included, along with formamide, the latter espe-

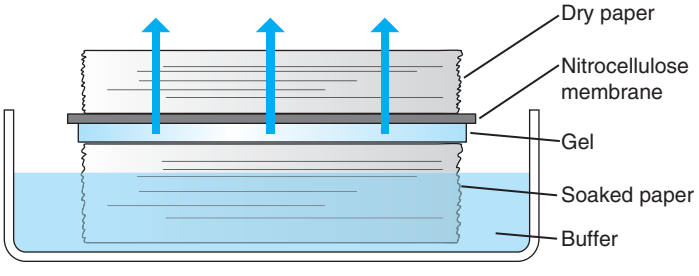
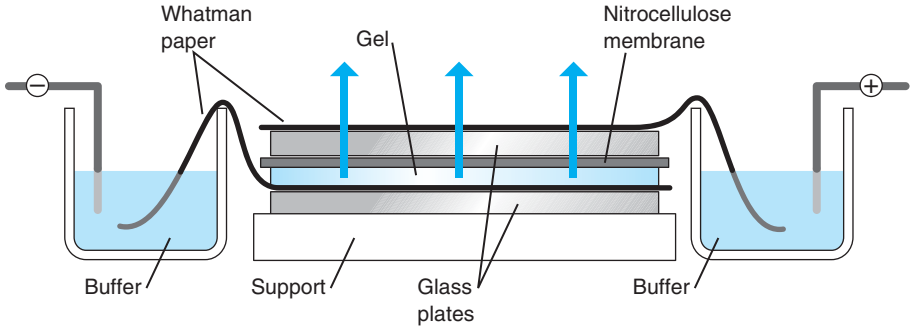


Figure 6-6 Capillary transfer. Driven by capillary movement of buffer from the soaked paper to the dry paper, denatured DNA moves from the gel to the membrane.



■ Figure 6-7 Electrophoretic transfer. This system uses electric current to mobilize the DNA from the gel to the membrane.

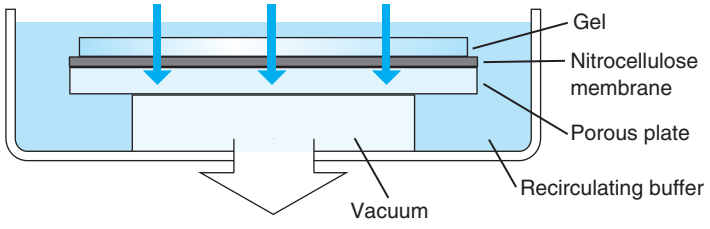
cially for RNA probes. The membrane is exposed to the prehybridization buffer at the optimal hybridization temperature for 30 minutes to several hours, depending on the specific protocol. At this stage the sample is ready for hybridization with the probe, which will allow visualization of the specific gene or region of interest.

Northern Blots

The **Northern blot** is a modification of the Southern blot technique and was designed to investigate RNA structure and quantity. Although most Northern analyses are performed to investigate levels of gene expression (transcription from DNA) and stability, the method can also be used to investigate RNA structural abnormalities resulting from aberrations in synthesis or processing, such as **alternate splicing**. Splicing abnormalities are responsible for a number of diseases, such as beta-thalassemias and familial isolated growth hormone deficiency. Analysis of RNA structure and quantity indirectly reveals mutations in the regulatory or splicing signals in DNA.

Care must be taken with RNA preparation to maintain an RNase-free environment. After isolation and quantitation of RNA, the samples (up to approximately 30 μg total RNA or 0.5–3.0 μg polyA RNA, depending on the relative abundance of the transcript under study) can be applied directly to agarose gels. Agarose concentrations of 0.8%-1.5% are usually employed. Polyacrylamide gels can also be used, especially for smaller transcripts; for instance, for analysis of viral gene expression.[2] Gel electrophoresis of RNA must be carried out under denaturing conditions for accurate transcript size assessment (see Chapter 5). Complete denaturation is also required for efficient transfer of the RNA from the gel to the membrane, as with the transfer of DNA in the Southern blot. Because the denaturation is carried out during electrophoresis, a separate denaturation step is not required for Northern blots. After electrophoresis, representative lanes can be cut from the gel, soaked in ammonium acetate to remove the denaturant, and stained with acridine orange or ethidium bromide to assess quality and equivalent sample loading (see Chapter 4).

Denaturant, such as formaldehyde, must be removed from the gel before transfer because it inhibits binding of the RNA to nitrocellulose. This is accomplished by rinsing the gel in de-ionized water. RNA is transferred in 10X or 20X SSC or 10X SSPE (1.8 M NaCl, 0.1 M sodium phosphate, pH 7.7, 10 mM EDTA) to nitrocellulose as



■ Figure 6-8 Vacuum transfer. This system uses suction to move the DNA out of the gel and onto the membrane.

Advanced Concepts

Some protocols call for destaining of the gel in sodium phosphate buffer (acridine orange) or 200 mM sodium acetate, pH 4.0 (ethidium bromide) before transfer. The latter destaining method may interfere with the movement of RNA from the gel during transfer. If formaldehyde has been used as a denaturant during electrophoresis, the gel must be rinsed and held in de-ionized water. Rinsing is not necessary if the formaldehyde concentration is less than 0.4 M.

described above for DNA. 20X SSC should be used for small transcripts (500 bases or less). The blotting procedure for RNA in the Northern blot is carried out in 20X SSC, similar to the procedure for DNA transfer in the Southern blot. Prehybridization and hybridization in formamide/SSC/SDS prehybridization/hybridization buffers are performed also as with Southern blot. If the RNA has been denatured in glyoxal, the membrane must be soaked in warm Tris buffer (65°C) to remove the denaturant immediately before prehybridization.

Western Blots

Another modification of the Southern blot is the **Western blot**.[3] The immobilized target for a Western blot is protein**.** There are many variations on Western blots. Generally, serum, cell lysate, or extract is separated on SDS-polyacrylamide gels (SDS-PAGE) or isoelectric focusing gels (IEF). The former resolves proteins according to molecular weight, and the latter according to charge. Dithiothreitol or 2-mercaptoethanol can also be used to separate proteins into subunits. Polyacrylamide concentrations vary 5%-20%. Depending on the complexity of the protein and the quantity of the target protein, 1–50 μg of protein is loaded per well. Before loading, the sample is treated with denaturant, such as mixing 1:1 with 0.04 M Tris HCl, pH 6.8, 0.1% SDS. The accuracy and sensitivity of the separation can be enhanced by using a combination of IEF gels followed by SDS-PAGE or by using two-dimensional gel electrophoresis. Prestained molecular weight standards are run with the samples to orient the membrane after transfer and to approximate the sizes of the proteins after probing. Standards ranging from 11,700 d (cytochrome C) to 205,000 d (myosin) are commercially available.

The gel system used may affect subsequent probing of proteins with antibodies. Specifically, denaturing gels could affect **epitopes** (antigenic sites on the protein) such that they will not bind with the labeled antibodies. Gel pretreatment with mild buffers such as 20% glycerol in 50 mM Tris-HCl, pH 7.4, can renature proteins before transfer.[4]

After electrophoresis, proteins can be blotted to membranes by capillary or electrophoretic transfer. Nitrocellulose has high affinity for proteins and is easily treated with detergent (0.1% Tween 20 in 0.05 M Tris and 0.15 M sodium chloride, pH 7.6) to prevent binding of the primary antibody to the membrane itself (**blocking**) before hybridization. Binding of proteins to nitrocellulose is probably hydrophobic as nonionic detergents can remove proteins from the membrane. Other membrane types that can be used for protein blotting are PVDF and anion (DEAE) or cation (CM) exchange cellulose.

Advanced Concepts

The Western blot method is used to confirm enzyme-linked immunoassay results for human immunodeficiency virus (HIV) and hepatitis C virus among other organisms. In this procedure, known HIV proteins are separated by electrophoresis and transferred and bound to a nitrocellulose membrane. The patient's serum is overlaid on the membrane, and antibodies with specificity to HIV proteins bind to their corresponding protein. Unbound patient antibodies are washed off, and binding of antibodies is detected by adding a labeled antihuman immunoglobulin antibody. If HIV antibodies are present in the patient's serum, they can be detected with antihuman antibody probes appearing as a dark band on the blot corresponding to the specific HIV protein to which the antibody is specific.

Probes

The probe for Southern and Northern blots is a single-stranded fragment of nucleic acid. The purpose of the probe is to identify one or more sequences of interest within a large amount of nucleic acid. The probe therefore should hybridize specifically with the target DNA or RNA that is to be analyzed. The probe can be RNA, denatured DNA, or other modified nucleic acids. **Peptide nucleic acids** (PNAs) and **locked nucleic acids** have also been used as probes. These structures contain normal nitrogen bases that can hybridize with complementary DNA or RNA, but the bases are connected by backbones different from the natural phosphodiester backbone of DNA and RNA. These modified backbones are resistant to nuclease degradation and, because of a reduced negative charge on their backbone, can hybridize more readily to target DNA or RNA.

Probes for Western blots are specific binding proteins or antibodies. A labeled secondary antibody directed against the primary binding protein is then used for the visualization of the protein band of interest.

DNA Probes

DNA probes are created in several ways. A fragment of the gene to be analyzed can be cloned on a bacterial plasmid and then isolated by restriction enzyme digestion and gel purification. The fragment, after labeling (see below) and denaturation, can then be used in Southern or Northern blot procedures.

Other sources of DNA probes include the isolation of a sequence of interest from viral genomes and in vitro organic synthesis of a piece of nucleic acid that has a particular sequence. The latter is used only for short, oligomeric probes. Probes can also be synthesized using the polymerase chain reaction (PCR) (see Chapter 7) to generate large amounts of specific DNA sequences.

The length of the probe will, in part, determine the specificity of the hybridization reaction. Probe lengths range from tens to thousands of base pairs. In analysis of the entire genome in a Southern blot, longer probes are more specific for a DNA region because they must match a longer sequence on the target. Shorter probes are not usually used in Southern blots because short sequences are more likely to be found in multiple locations in the genome, resulting in high background binding to sequences not related to the target region of interest. Short probes are more appropriate for mutational analysis as they are sensitive to single base mismatches (see Chapter 8). The probe is constructed so that it has a complementary sequence to the targeted gene. In order to bind to the probe then, the target nucleic acid has to contain the sequence of interest. There are typically fewer copies of a specific sequence in the genome, and therefore only a few bands will be apparent after detection.

Properly prepared and stored DNA probes are relatively stable and easy to manufacture. Double-stranded DNA probes must be denatured before use. This is usually accomplished by heating the probe (e.g., 95°C, 10–15 min) in hybridization solution or treating with 50% formamide/2X SSC at a lower temperature for a shorter time (e.g., 75°C, 5–6 min)

RNA Probes

RNA probes are often made by transcription from a synthetic DNA template in vitro. These probes are similar to DNA probes with equal or greater binding affinity to homologous sequences. Because RNA and DNA form a stronger helix than DNA/DNA, the RNA probes may offer more sensitivity than DNA probes in the Southern blot.

RNA probes can be synthesized directly from a plasmid template or from template DNA produced by PCR (see Chapter 7). Predesigned systems are commercially available for this purpose. These products include plasmid vector DNA such as pGEM (Promega) or pBluescript (Stratagene), containing a binding site for RNA polymerase (promoter) and a cloning site for the sequences of interest, and a DNA-dependent RNA polymerase from *Salmonella* bacteriophage SP6 or *E. coli* bacteriophage T3 or T7. DNA sequences complementary to the RNA transcript to be analyzed are cloned into the plasmid vector using restriction enzymes. The recombinant vector containing the gene of interest is then linearized, and the RNA probe is transcribed in vitro from the promoter. RNA probes are labeled by incorporating a radioactive or modified nucleotide during the in vitro transcription process.

Either coding or complementary RNA will hybridize to a double-stranded DNA target. Care must be taken,

however, in designing RNA probes for Northern blots. The complementary sequence to the target must be used for the probe. A probe of identical sequence to the target RNA (coding sequence) will not hybridize. Because of labeling during synthesis, RNA probes can have a high specific activity (signal to micrograms of probe) that increases the sensitivity of the probe. To avoid high background, some protocols include digestion of nonhybridized RNA, using a specific RNase, such as RNase A, after hybridization is complete.

RNA probes are generally less stable than DNA probes and cannot be stored for long periods. Synthesis of an RNA probe by transcription from a stored template is relatively simple and should be performed within a few days of use. The DNA template can be removed from the probe by treatment with RNase-free DNase. Although RNA is already single-stranded, denaturation before use is recommended in order to eliminate secondary structure internal to the RNA molecule.

Other Nucleic Acid Probe Types

Peptide nucleic acid and locked nucleic acid probes (Fig. 6-9 and 6-10) can be synthesized using chemical methods.[5–8] These modified nucleic acids have the advantage of being resistant to nucleases that would degrade DNA and RNA by breaking the phosphodiester backbone. Further, the negative charge of the phosphodiester backbones of DNA and RNA counteract hydrogen bonding between the bases of the probe and target sequences. Structures such as PNA that do not have a negative charge hybridize more efficiently.

Advanced Concepts

These structures are not only useful in the laboratory, they are also potentially valuable in the clinic. Several structures have been proposed for use in antisense gene therapy Fig. 6-10. Introduction of sequences complementary to messenger RNA of a gene (antisense sequences) will prevent translation of that mRNA and expression of that gene. If this could be achieved in whole organisms, selected aberrantly expressed genes or even viral genes could be turned off. One drawback of this technology is the degradation of natural RNA and DNA by intracellular nucleases. The nuclease-resistant structures are more stable and available to hybridize to the target mRNA.

Protein Probes

Western blot protein probes are antibodies that bind specifically to the immobilized target protein. Polyclonal or monoclonal antibodies can be used for this purpose. **Polyclonal antibodies** are made by immunization with a specific antigen, usually a peptide or protein. Small molecules (**haptens**) attached to protein carriers, carbohydrates, nucleic acids, and even to whole cells and tissue extracts can be used to generate an antibody response. Adjuvants, such as Freund's adjuvant, are used to enhance the antibody titer by slowing the degradation of the protein and lengthening the time the immune system is

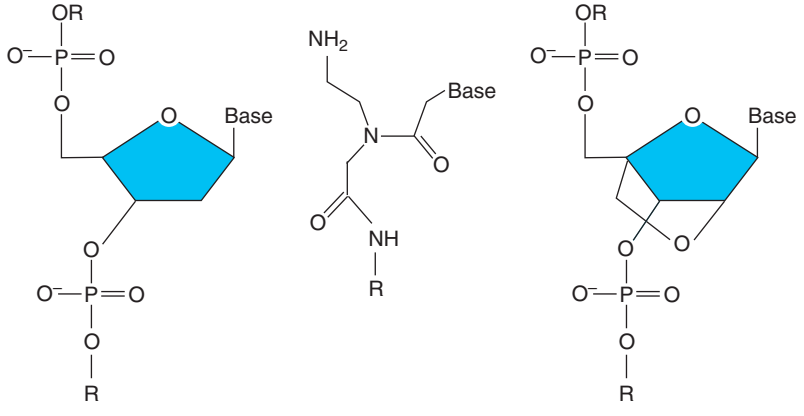


Figure 6-9 Peptide nucleic acids have the phosphodiester bond (left) replaced with carbon nitrogen peptide bonds (center). Locked nucleic acids are bicyclic nucleoside monomers where the ribose sugar contains a methylene link between its 2′ oxygen and 4′ carbon atoms (right).

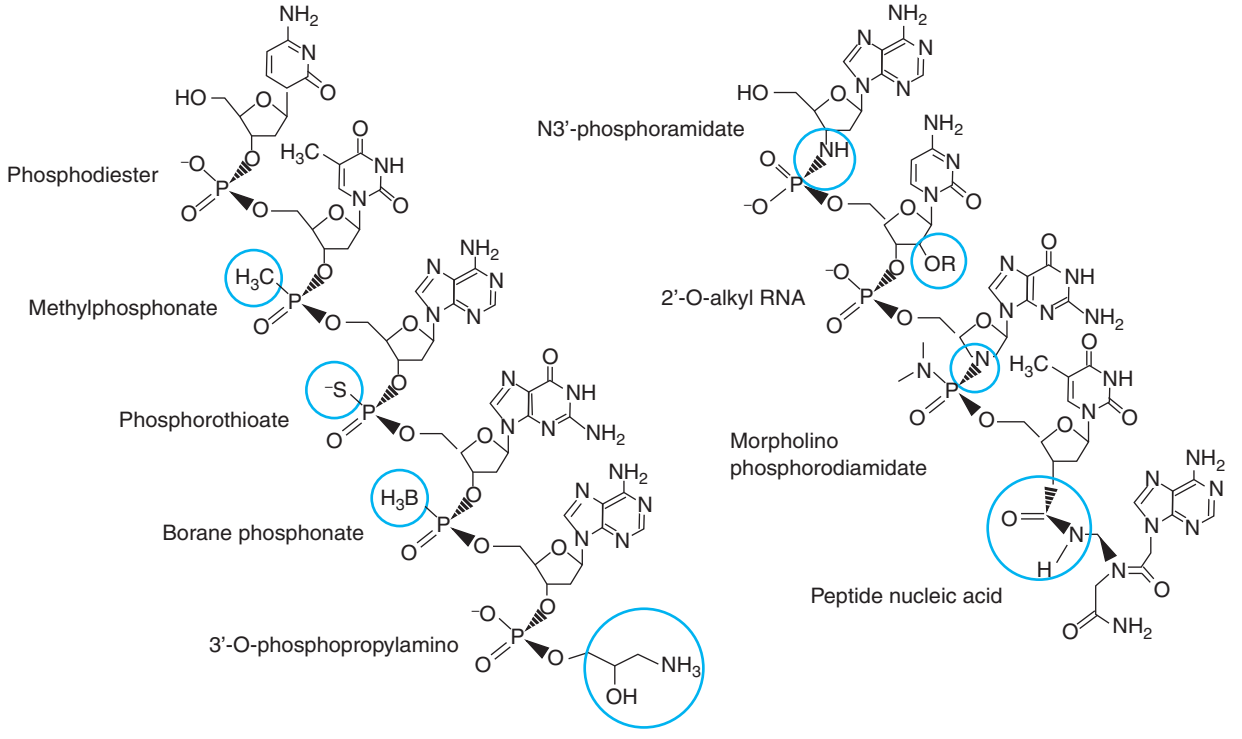


Figure 6-10 Modifications of the phosphodiester backbone of nucleic acids. [28, 29]

exposed to the stimulating antigen. The immunoglobulins are subsequently isolated from sera by affinity chromatography.

Polyclonal antibodies are a mixture of immunoglobulins that are directed at more than one epitope (molecular structure) on the antigen. Monoclonal antibodies are more difficult to produce. Kohler and Milstein first demonstrated that spleen cells from immunized mice could be fused with mouse myeloma cells to form hybrid cells (**hybridomas**) that could grow in culture and secrete antibodies.[9] By cloning the hybridomas (growing small cultures from single cells), preparations of specific antibodies could be produced continuously. The clones could then be screened for antibodies that best react with the target antigen. Monoclonal antibodies can be isolated from cell culture fluid. Higher titers of antibodies are obtained by inoculating the antibody-producing hybridoma into mice and collecting the peritoneal fluid. The monoclonal antibody is then isolated by chromatography. Polyclonal antibodies are useful for immunoprecipitation methods and can be used for Western blots. With their greater specificity, monoclonal antibodies can be used for almost any procedure.

In Western blot technology, polyclonal antibodies can give a more robust signal, especially if the target epitopes are partially lost during electrophoresis and transfer. Monoclonal antibodies are more specific and may give less background; however, if the targeted epitope is lost, these antibodies do not bind, and no signal is generated. Dilution of primary antibody can range from 1/100 to 1/100,000, depending on the sensitivity of the detection system (see below).

Probe Labeling

In order to visualize the probe bound to target fragments on the blot, the probe must be **labeled** and generate a detectable signal. The original Southern analyses used radioactive labeling with ^{32}P. This labeling was achieved by introduction of nucleotides containing radioactive phosphorus to the probe. Today, many clinical laboratories use nonradioactive labeling to avoid the hazard and

expense of working with radiation. Nonradioactive labeling methods are based on indirect detection of a tagged nucleotide incorporated in or added to the probe. The two most commonly used nonradioactive tags are **biotin** and **digoxygenin** (Fig. 6-11), either of which can be attached covalently to a nucleotide triphosphate, usually UTP or CTP.

There are three basic methods that are used to label a DNA probe: end-labeling, nick translation, and random priming. **End-labeling** involves the addition of labeled nucleotides to the end of the fragment using terminal transferase or T4 polynucleotide kinase. In **nick translation**, the labeled nucleotides are inserted into the fragment at single-stranded breaks, or nicks, in a double-stranded probe. DNA polymerase extends the broken end of one strand using the intact complementary strand for a template and displaces the previously hybridized strand. **Random priming** generates new single-stranded versions of the probe with the incorporation of the labeled nucleotides. The synthesis of these new strands is primed by oligomers of random sequences that are six to ten bases in length. These short sequences will, at some frequency, complement sequences in the probe and prime synthesis of a copy of the probe with incorporated labeled nucleotides.

RNA probes are transcribed from cloned DNA or amplified DNA. These probes are labeled during their synthesis by the incorporation of radioactive, biotinylated, or digoxygenin-tagged nucleotides. Unlike double-stranded complementary DNA probes and targets that contain both strands of the complementary sequences, RNA probes are single-stranded with only one strand of the complementary sequence represented.

Nucleic Acid Probe Design

The most critical parts of any hybridization procedure are the design and optimal hybridization of the probe, which determines the specificity of the results. With nucleic acids, the more optimal the hybridization conditions for a probe/target interaction, the more specific the probe. Longer probes (500–5000 bp) offer greater specificity with decreased background, but they may be difficult or expensive to synthesize. Long probes are less affected by point mutations or polymorphisms within the sequence targeted by the probe or within the probe itself.

Shorter probes (<500 bp) are less specific than longer ones in Southern blotting applications. A short sequence has a higher chance of being repeated randomly in unrelated regions of the genome. Short probes are ideal, how-

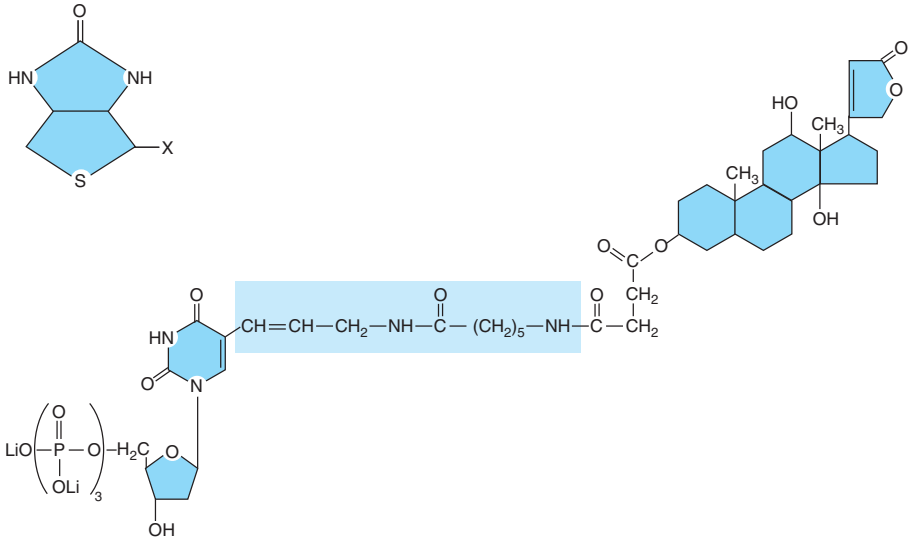
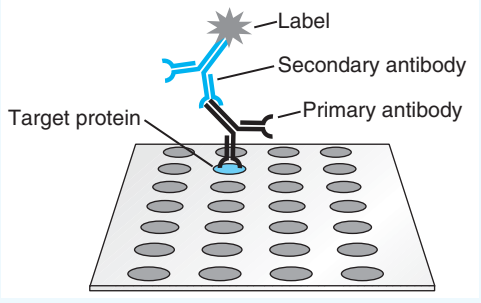


Figure 6-11 Biotin (top) has a variable side chain (X). The polycyclic digoxygenin (bottom) is shown covalently attached to UTP (dig-11-UTP). This molecule can be covalently attached or incorporated into DNA or RNA to make a labeled probe.

Advanced Concepts

Protein probes used in Western blot can be covalently bound to an enzyme, usually horseradish peroxidase or alkaline phosphatase. Unconjugated antibodies can be detected after binding with a conjugated secondary antibody to the primary probe, such as mouse antihuman or rabbit antimouse antibodies (Fig 6-12). The secondary antibodies will recognize any primary antibody by targeting the Fc region.



■ Figure 6-12 Probe binding to Western blots may include an unlabeled primary antibody that is subsequently bound by a secondary antibody carrying a label for detection.

ever, for mutation analysis, as their binding affinity is sensitive to single base pair changes within a target binding sequence.

The sequence of the probe can affect its binding performance as well. A sequence with numerous internal complementary sequences will fold and hybridize with itself, which will compete with hybridization to the intended target. The probe folding or secondary structure is especially strong in sequences with high GC content, decreasing the binding efficiency to the target sequence.

Hybridization Conditions, Stringency

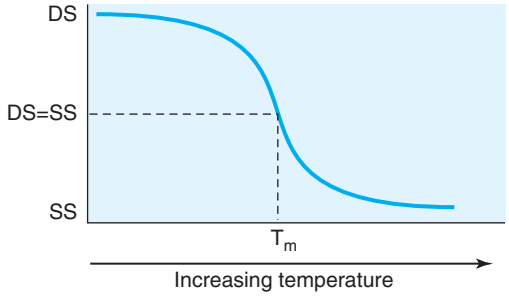
Southern blot and Northern blot probing conditions must be empirically optimized for each nucleic acid target. **Stringency** is the combination of conditions in which the target is exposed to the probe. Conditions of high stringency are more demanding of probe:target complementarity. Low stringency conditions are more forgiving. If conditions of stringency are set too high, the probe will not bind to its target. If conditions are set too low, the probe will bind unrelated targets, complicating interpretation of the final results.

Several factors affect stringency. These include temperature of hybridization, salt concentration of the hybridization buffer, and the concentration of denaturant such as formamide in the buffer. The nature of the probe sequence can also impinge on the level of stringency. A probe with a higher percentage of G and C bases will bind under more stringent conditions than one with greater numbers of A and T bases. The ideal hybridization conditions can be estimated from calculation of the **melting temperature**, or T_m, of the probe sequence. The T_m is a way to express the amount of energy required to separate the hybridized strands of a given sequence (Fig. 6-13). At the T_m, half of the sequence is double-stranded, and half is single-stranded. The T_m for a double-stranded DNA sequence in solution is calculated by the following formula:

$$T_m = 81.5°C + 16.6 \log M + 0.41 (\%G + C) - 0.61 (\%\ \text{formamide}) - (600/n)$$

where M = sodium concentration in mol/L and n = number of base pairs in the shortest duplex.

RNA:RNA hybrids are more stable than DNA:DNA hybrids due to less constraint by the RNA phosphodiester



■ Figure 6-13 Melting temperature, Tm, is the point at which exactly half of a double-stranded sequence becomes single-stranded. The melting temperature is determined at the inflection point of the melt curve. DS, double-stranded; SS, single-stranded.

backbone. The formulas, therefore, are slightly different. For RNA:RNA hybrids the formula is:

$$T_m = 79.8°C + 18.5 \log M + 0.58 (\%G + C) + 11.8 (\%G + C) - 0.35 (\% \text{ formamide}) - (820/n)$$

DNA:RNA hybrids have intermediate affinity:

$$T_m = 79.8°C + 18.5 \log M + 0.58 (\%G + C) + 11.8 (\%G + C) - 0.50 (\% \text{ formamide}) - (820/n)$$

The T_m is also a function of the extent of complementarity between the sequence of the probe and that of the target sequence. For each 1% difference in sequence, the T_m decreases 1.5°C. Furthermore, the T_m of RNA probes is higher. RNA:DNA hybrids increase T_m by 10°–15°C. DNA:DNA hybrids increase T_m by 20°−25°C.

The T_m for short probes (14–20 bases) can be calculated by a simpler formula:

$$T_m = 4°C \times \text{number of GC pairs} + 2°C \times \text{number of AT pairs}$$

The hybridization temperature of oligonucleotide probes is about 5°C below the melting temperature. The effect of sequence complexity on hybridization efficiency can be illustrated by the **$C_o t$** value. **Sequence complexity** is the length of unique (nonrepetitive) nucleotide sequences. After denaturation, complex sequences require more time to reassociate than simple sequences, such as polyA:polyU. $C_o t$ is an expression of the sequence complexity (Fig. 6-14). $C_o t$ is equal to the initial DNA concentration (C_o) times the time required to reanneal (t). **$Cot_{1/2}$** is the time required for half of a double-stranded sequence to anneal under a given set of conditions.

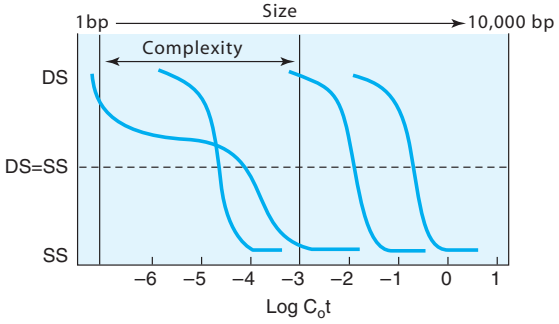


Figure 6-14 Reannealing of single-stranded (SS) DNA to double-stranded (DS) DNA vs. time at a constant concentration yields a sigmoid curve. The complexity of the DNA sequence will widen the sigmoid curve. Increasing the length of the double-stranded DNA will shift the curve to the right.

Advanced Concepts

$C_o t$ was used to demonstrate that mammalian DNA consisted of sequences of varying complexity. Britten and Kohne[24] used *Eschericia coli* and calf thymus DNA to demonstrate this. When they measured reassociation of *E. coli* DNA vs. time, a sigmoid curve was observed, as expected for DNA molecules with equal complexity. In comparison, the calf DNA reassociation was multifaceted and spanned several orders of magnitude (see Fig. 6-14). The spread of the curve results from the mixture of slowly renaturing unique sequences and rapidly renaturing repeated sequences (satellite DNA).

T_m and $C_o t$ values can provide a starting point for optimizing stringency conditions for Southern blot analysis. Hybridization at a temperature 25°C below the T_m for 1–3 $C_o t_{1/2}$ is considered optimal for a double-stranded DNA probe. Final conditions must be established empirically, especially for short probes. Stringency conditions for routine analyses, once established, will be used for all subsequent assays. In the event a component of the procedure is altered, new conditions may have to be established.

Hybridizations are generally performed in hybridization bags or in glass cylinders. Within limits, the sensitivity of the analysis increases with increased probe concentration. Because the probe is the limiting reagent, it is practical to keep the volume of the hybridization solution low. The recommended volume of hybridization buffer is approximately 10 mL/100 cm^2 of membrane surface area.

Formamide in the hybridization buffer effectively lowers the optimal hybridization temperature. This is especially useful for RNA probes and targets that, because of secondary structure, are more difficult to denature and tend to have a higher renaturation (hybridization) temperature. Incubation of the hybridization system in sealed bags in a water bath or in capped glass cylinders in rotary ovens maintains the blot at the proper temperature.

Short probes (<20 bases) can hybridize in 1–2 hours. In contrast, longer probes require much longer hybridiza-

Advanced Concepts

The nature of the probe label will affect hybridization conditions. Unlike ^{32}P labeling, the bulky nonradioactive labels (see Fig. 6-12) disturb the hybridization of the DNA chain. The temperature of hybridization with these types of probes will be lower than that used for radioactively labeled probes.

tion times. For Southern and Northern blots with probes >1000 bases in length, incubation is carried out for 16 hours or more. Raising the probe concentration can increase the hybridization rates. Also, inert polymers, such as dextran sulfate, polyethylene glycol, or polyacrylic acid, accelerate the hybridization rates for probes longer than 250 bases.

Detection Systems

This chapter has so far addressed the transfer of electrophoresed DNA, RNA, or protein to a solid membrane support and hybridization or binding of a specific probe to the target sequence of interest. The next step in these procedures is to detect whether the probe has bound to the target molecule and, if it has bound, the relative location of the binding. The original ^{32}P-labeled probes offered the advantages of simple and sensitive detection.

Advanced Concepts

Optimization may not completely eliminate all nonspecific binding of the probe. This will result in extra bands in control lanes or cross-hybridizations. At a given level of stringency, any increase to eliminate cross-hybridization will lower the binding to the intended sequences. It becomes a matter of balancing the optimal probe signal with the least amount of non-target binding. Cross-hybridizations are usually recognizable as bands of the same size in multiple runs. It is important to take cross-hybridization bands into account in the final interpretation of the assay results.

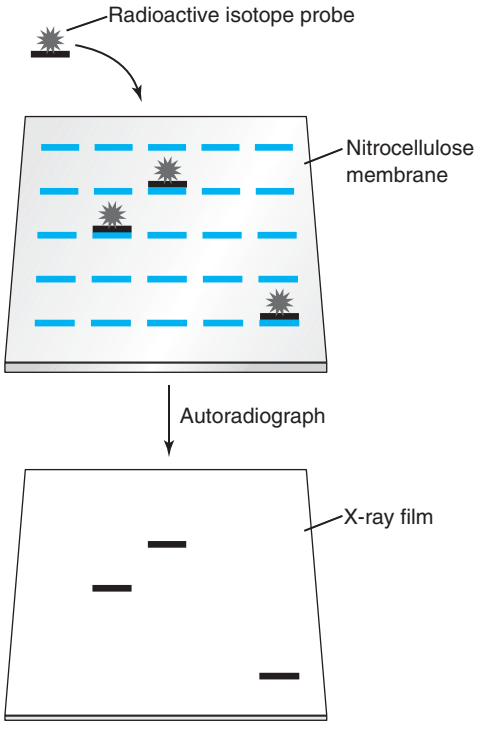


Figure 6-15 A DNA or RNA probe labeled with radioactive phosphorous atoms (^{32}P or ^{33}P) hybridized to target (homologous) sequences on a nitrocellulose membrane. The fragments to which the probe is bound can be detected by exposing autoradiography film to the membrane.

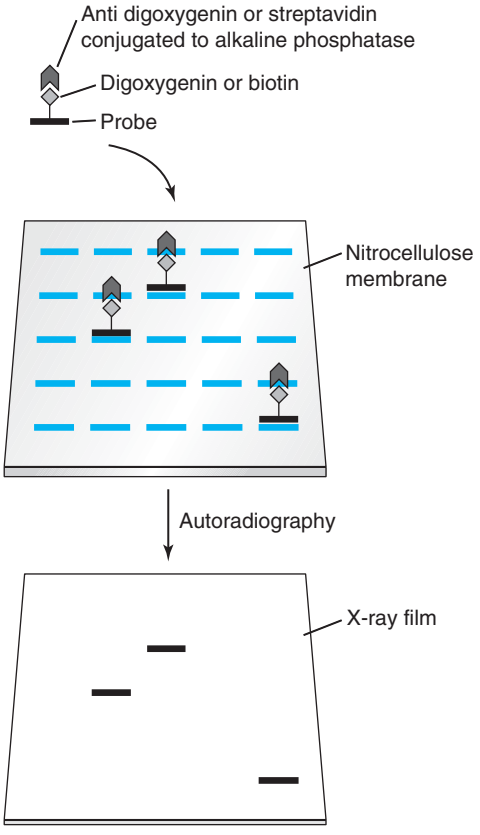
After hybridization, unbound probe is washed off, and the blot is exposed to light-sensitive film to detect the fragments that are hybridized to the radioactive probe (Fig. 6-15). Wash conditions must be formulated so that only completely hybridized probe remains on the blot. Typically wash conditions are more stringent than those used for hybridization.

Nonradioactive detection systems require a more involved detection procedure. For most nonradioactive systems, the probe is labeled with a nucleotide covalently attached to either digoxygenin or biotin. The labeled nucleotide is incorporated into the nucleotide chain of the probe by in vitro transcription, nick translation, primer extension, or addition by terminal transferase. Digoxygenin- or biotin-labeled probe is incubated together with the blot with sample(s) containing the target sequence of interest to allow for hybridization to occur. After hybridization, unbound probe is washed away. Then, antidigoxygenin

Advanced Concepts

In addition to CSPD and CDP-star (Roche Diagnostics Corp.), there are several substrates for chemiluminescent detection that are 1–2 dioxetane derivatives such as 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy) phenyl-1,2-dioxetane (AMPPD; Tropix, Inc.). Dephosphorylation of these compounds by the alkaline phosphatase conjugate bound to the probe on the membrane results in a light-emitting product (see Fig. 6-17).[25] Other luminescent molecules include acridinium ester and acridinium (N-sulfonyl) carboxamide labels, isoluminol, and electrochemiluminescent ruthenium tris-bipyridyl labels.

The substrate used most often for chromogenic detection is a mixture of Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Upon dephosphorylation of BCIP by alkaline phosphatase, it is oxidized by NBT to give a dark blue indigo dye as an oxidation product. BCIP is reduced in the process, and also yields a blue product (see Fig. 6-18).



■ Figure 6-16 Indirect non-radioactive detection. The probe is covalently attached to digoxygenin or biotin. After hybridization, the probe is bound by antibodies to digoxygenin or streptavidin conjugated to alkaline phosphatase (AP). This complex is exposed to color or light producing substrates of AP producing color on the membrane or light detected with autoradiography film.

antibody or streptavidin, respectively, conjugated to alkaline phosphatase (AP conjugate, Fig. 6-16) is added to reaction mix to bind to the digoxigenin- or biotin-labeled probe:target complex. Horseradish peroxidase (HRP) conjugates can also be used in this procedure. After the binding of the conjugate, the membrane is bathed in a solution of substrate that, when oxidized by HRP or dephosphorylated by AP, produces a signal. Substrates frequently used are dioxetane or tetrazolium dye derivatives, which generate chemiluminescent (Fig. 6-17) or chromogenic (Fig. 6-18) signals, respectively (Table 6.2).

As with radioactive detection, the chemiluminescent signal produced by the action of the enzyme on dioxetane develops in the dark by autoradiography. Light released by phosphorylation of dioxetane takes place at the location on the membrane where the probe is bound and darkens the light-sensitive film. Chemiluminescent detection is often stronger and develops faster than radioactive detection. A disadvantage of chemiluminescent detection is that it is harder to control and sometimes produces high backgrounds. New substrates have been designed to minimize these drawbacks. Unlike radioactive detection, in which testing the membrane with a Geiger counter can give an indication of how "hot" the bands are and consequently how long to expose the membrane to the film, chemiluminescent detection may require developing films at different intervals to determine the optimum exposure time.

For chromogenic detection, a colored signal is produced when the enzyme interacts with a derivative of a tetrazolium dye and is detected directly on the membrane filter. The advantage of this type of detection is that the color can be observed as it develops and the reaction stopped at a time when there is an optimum signal-to-background ratio. In general, chromogenic detection is

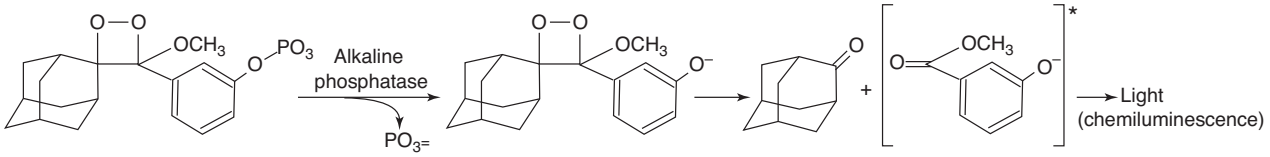


Figure 6-17 Light is emitted from 1-2 dioxetane substrates after dephosphorylation by alkaline phosphatase to an unstable structure. This structure releases an excited anion that emits light.

not as sensitive as chemiluminescent detection and can also result in a higher background, especially with probes labeled by random priming.

The key to a successful blotting method is a high **signal-to-noise** ratio. Ideally, the probe and detection systems should yield a specific and robust signal. High specific signal, however, may be accompanied by high background (noise). Therefore, sensitivity of detection is sometimes sacrificed to generate a more specific signal.

Interpretation of Results

When a specific probe binds to its target immobilized on a membrane, the binding is detected as described in the previous section, with the end result being the visualization of a **"band"** on the membrane or film. A band is simply seen as a line running across the width of the lane. Analysis of bands, i.e., presence or absence or location in the lane, produced by Southern blot can be straightforward or complex, depending on the sample and the design of the procedure. Figure 6-19 is a depiction of a Southern blot result. The bands shown can be visualized either on a membrane or on an autoradiographic film. If a gene locus has a known restriction pattern, for instance in lane 1, then samples can be tested to compare their restriction patterns. In the figure, the sample in lane 3 has the identical pattern, i.e., both lanes have the same number of bands, and the bands are all in the same location on the gel and are likely to be very similar if not identical in sequence to the sample in lane 1. Southern blot

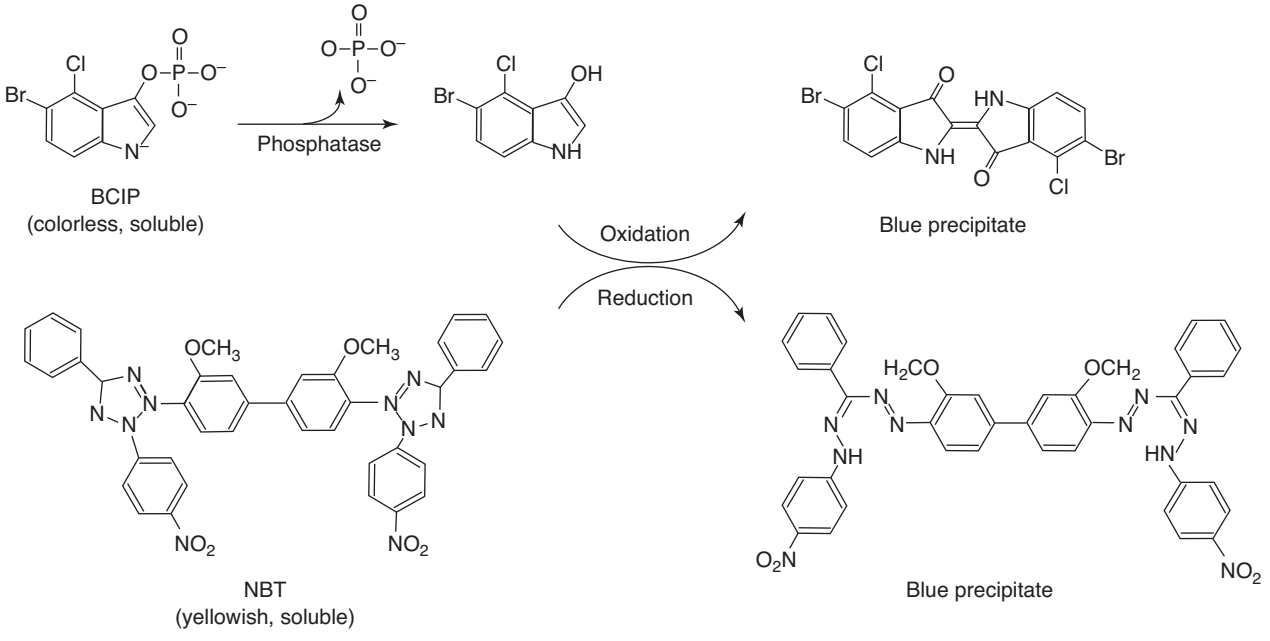


Figure 6-18 Generation of color with BCIP and NBT. Alkaline phosphatase dephosphorylates BCIP which then reduces NBT making an insoluble blue precipitate.

Table 6.2 **Nonradioactive Detection Systems**

Type of Detection	Enzyme	Reagent	Reaction Product
Chromogenic	HRP	4-chloro-1-naphthol (4CN)	Purple precipitate
	HRP	3,3′-diaminobenzidine	Dark brown precipitate
	HRP	3,3′,5,5′,-tetramethylbenzidine	Dark purple stain
	Alkaline phosphatase	5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium	Dark blue stain
Chemiluminescent	HRP	Luminol/H_2O_2/p-iodophenol	Blue light
	Alkaline phosphatase	1–2 dioxetane derivatives	Light
	Alkaline phosphatase	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}]decan} 4-yl)-1-phenyl phosphate and derivatives (CSPD, CDP-Star)	Light

cannot detect tiny deletions or insertions of nucleotides or single nucleotide differences unless they affect a specific restriction site. For some assays, cross-hybridization may confuse results. These artifacts can be identified by their presence in every lane at a constant size.

Northern (or Western) blots are usually used for analysis of gene expression, although they can also be used to analyze transcript size, transcript processing, and protein modification. For these analyses, especially when estimating expression, it is important to include an internal control to correct for errors in isolation, gel loading, or transfer of samples. The amount of expression is then determined relative to the internal control (Fig. 6-20). In the example shown, the target transcript or protein product is expressed in increasing amounts, left to right. The internal standardized control (lower band) assures that a sample has low expression of target transcript of protein product and that the low signal is not due to technical difficulties.

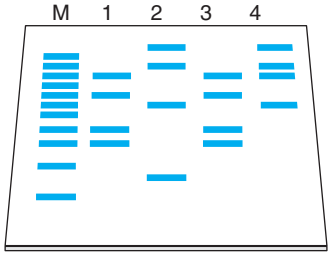


Figure 6-19 Example of a Southern blot result. The first lane (M) contains molecular weight markers. Restriction digests of a genetic region can be compared to determine differences in structure. Two samples with the same pattern (for example, lanes 1 and 3) can be considered genetically similar.

Array-Based Hybridization

Dot/Slot Blots

There are many variations on Southern hybridization methods. In cases where the determination of the size of the target is not required, DNA and RNA can be more quickly analyzed using dot blots or slot blots. These procedures are usually applied to expression, mutation, and amplification/deletion analyses.

For dot or slot blots, the target DNA or RNA is deposited directly on the membrane. Various devices, some

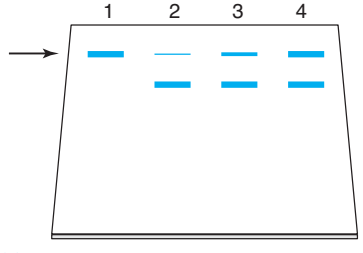


Figure 6-20 Example of a Northern or Western blot result. Lane 1 contains a positive control transcript or protein (arrow) to verify the probe specificity and target size. Molecular weight markers can also be used to estimate size as in Southern analysis. The amount of gene product (expression level) is determined by the intensity of the signal from the test samples relative to a control gene product (lower band in lanes 2–4). The control transcript is used to correct for any differences in isolation or loading from sample to sample.

with vacuum systems, have been designed to deposit the target on the membrane. A pipet can be used for procedures testing only a few samples. For **dot blots**, the target is deposited in a circle or dot. For **slot blots**, the target is deposited in an oblong bar (Fig. 6-21). Slot blots are more accurate for quantitation by densitometry scanning because they eliminate the error that may arise from scanning through a circular target. If the diameter of the scanned area is not exactly the same from one sample to another, comparative results may be inaccurate. Dot blots are useful for multiple qualitative analyses where many targets are being compared, such as mutational analyses.

Dot and slot blots are performed most efficiently on less complex samples, such as PCR products or selected mRNA preparations. Without gel resolution of the target fragments, it is important that the probe hybridization conditions are optimized for these types of blots because cross-hybridizations cannot be definitively distinguished from true target identification. A negative control (DNA of equal complexity but without the targeted sequence) serves the baseline for interpretation of these assays. When performing expression analysis by slot or dot blots, it is also important to include an amplification or normalization control, as shown on the right in Figure 6-21. This allows correction for loading or sample differences. This control can also be analyzed on a separate duplicate membrane to avoid cross-reactions between the test and control probes.

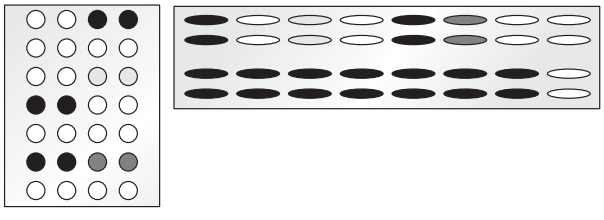


Figure 6-21 Example configuration of a dot blot (left) and a slot blot (right). The target is spotted in duplicate, side by side, on the dot blot. The last two rows of spots contain positive, sensitivity and negative control followed by a blank with no target. The top two rows of the slot blot gel on the left represent four samples spotted in duplicate, with positive, sensitivity and negative control followed by a blank with no target in the last four samples on the right. The bottom two rows represent a loading or normalization control that is often useful in expression studies to confirm that equal amounts of DNA or RNA were spotted for each test sample.

Genomic Array Technology

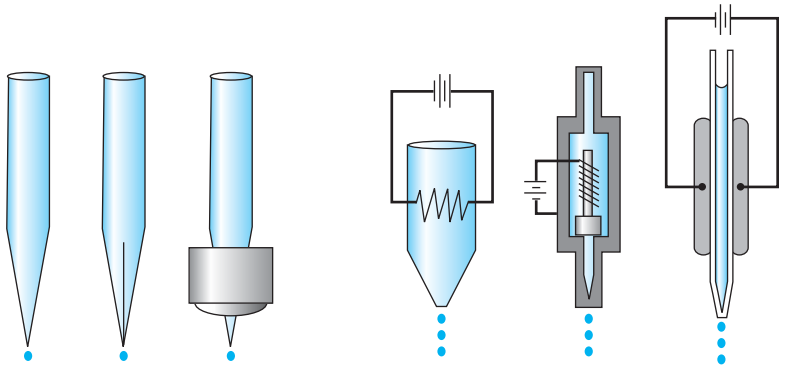
Array technology can be applied to gene (DNA) analysis by performing comparative genome hybridization and to gene expression (RNA or protein) analysis on expression arrays. There are several types of array technologies, including macroarrays, microarrays, high density oligonucleotide arrays, and microelectronic arrays.

Macroarrays

In contrast to Northern and Southern blots, dot (and slot) blots offer the ability to test and analyze larger numbers of samples at the same time. These methodologies are limited, however, by the area of the substrate material, nitrocellulose membranes, and the volume of hybridization solution required to provide enough probe to produce an adequate signal for interpretation. In addition, although up to several hundred test samples can be analyzed simultaneously, those samples can be tested for only one gene or gene product. A variation of this technique is the **reverse dot blot**, in which several different probes are immobilized on the substrate, and the test sample is labeled for hybridization with the immobilized probes. In this configuration, the terminology can be confusing. Immobilized probe is sometimes referred to as the target, and the labeled specimen DNA, RNA, or protein is called the probe. Regardless of the designation, the general idea is that a known sequence is immobilized at a known location on the blot, and the amount of sample that hybridizes to it is determined by the signal from the labeled sample.

Reverse dot blots on nitrocellulose membranes of several to several thousand targets are **macroarrays**. Radioactive or chemiluminescent signals are typically used to detect the hybridized targets in the sample. Macroarrays are created by spotting multiple probes onto nitrocellulose membranes. The hybridization of labeled sample material is read by eye or with a phosphorimager (a quantitative imaging device that uses storage phosphor technology instead of x-ray film). Analysis involves comparison of signal intensity from test and control samples spotted on duplicate membranes.

Although macroarrays greatly increase the capacity to assess numerous targets, this analysis system is still limited by the area of the membrane and the specimen requirements. As the target number increases, the volume of sample material required increases. This limits the utility of this method for use on small amounts of test mate-



■ Figure 6-22 Pen-type (left) and ink-jet (right) technologies used to spot arrays.

rial, especially as might be encountered with clinical specimens.

Microarrays

In 1987, the use of treated glass instead of nitrocellulose or nylon membranes for the production of arrays was developed, increasing the versatility of array applications. With improved spotting technology and the ability to deposit very small target spots on glass substrates, the macroarray evolved into the **microarray**. Tens of thousands of targets can be screened simultaneously in a very small area by miniaturizing the deposition of droplets (Fig. 6-22). Automated depositing systems (arrayers) can place more than 80,000 spots on a glass substrate the size of a microscope slide. The completion of the rough draft of the human genome sequence revealed that the human genome may consist of fewer than 30,000 genes. Thus, even with spotting representative sequences of each gene in triplicate, simultaneous screening of the entire human genome on a single chip is within the scope of array technology.

The larger nitrocellulose membrane, then, is replaced by a glass microscope slide. The slide carrying the array of targets is referred to as a **chip** (Fig. 6-23). Targets are usually DNA, either cDNAs, PCR products, or oligomers; however, targets can be DNA, RNA, or protein. Targets are spotted in triplicate and spaced across the chip to avoid any geographic artifacts that may occur from uneven hybridization or other technical problems. Probes are usually cDNA-generated from sample RNA but can, as well, be genomic DNA, RNA, or protein.

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The first automated arrayer was described in 1995 by Patrick Brown at Stanford University.[26] This and later versions of automated arrayers use pen-type contact to place a dot of probe material onto the substrate. Modifications of this technology include the incorporation of ink jet printing systems to deposit specific targets at designated positions using thermal, solenoid, or piezoelectric expulsion of target material (see Fig. 6-22).

Advanced Concepts

The analysis of the entire genome or sets of related genes is the relatively new field of **genomics**. Knowing the combinatorial and interrelated functions of gene products, observation of the behavior of sets of genes or genomes, is a more accurate method for analyzing biological states or responses. Stanley Fields[27] predicted that the entire collection of proteins coded by the genome, known as the **proteome**, is likely to be ten times more complex than the genome. The study of entire sets of proteins, or **proteomics**, will also be facilitated by array technology using antigen/antibody or receptor/ligand binding in the array format. Mass spectrometry can also be applied to the study of proteomics.

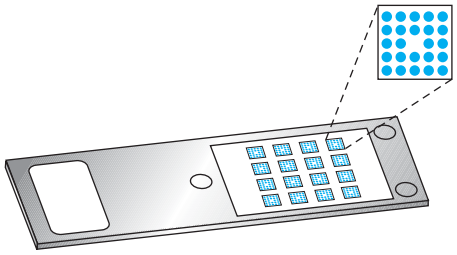

Figure 6-23 Microarray, or DNA chip, is a glass slide carrying 384 spots. Arrays are sometimes supplied with fluorescent nucleotides for use in labeling the test samples, and software for identification of the spots on the array by the array reader.

Other methods used to deposit targets on chips include performing DNA synthesis directly on the glass or silicon support.[10] This technique uses sequence information to design oligonucleotides and to selectively mask, activate, and covalently attach nucleotides at designated positions on the chip. Proprietary photolithography techniques (Affymetrix) allow for highly efficient synthesis of short oligomers (10–25 bases long) on high-density arrays (Fig. 6-24). These oligomers can then be probed with labeled fragments of the test sequences. Using this technology, more than 100,000 targets can be applied to chips. These types of arrays are called **high-density oligonucleotide arrays** and are used for mutation analysis, single nucleotide polymorphism analysis, and sequencing.

Another type of array method uses microelectronics to focus targets to specific positions on the array (Fig. 6-25). Once bound, these targets can be hybridized to labeled DNA or RNA samples under controlled conditions. These are **microelectronic arrays**. In this technology, each position on the array is attached to an electrode

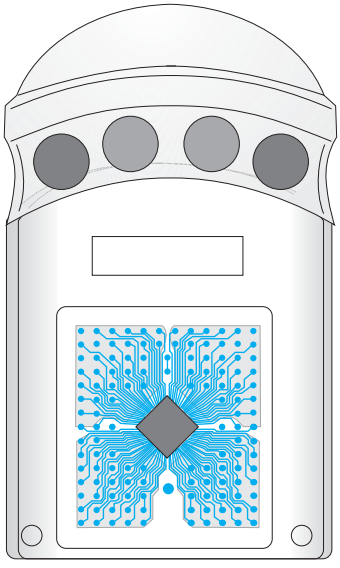

Figure 6-25 Microelectronic chip with a ten by ten array (center diamond). Each of the 100 stations on the array is attached to a separate electrode (Nanogen).

that can be programmed to attract and concentrate the labeled sample. By enhancing the hybridization conditions separately at each spot on the array, single nucleotide resolution is possible even on longer sample fragments.

Sample preparation for array analysis requires fluorescent labeling of the test sample as microarrays and other high-density arrays are read by automated fluorescent detection systems. The most frequent labeling method used for RNA is synthesis of cDNA or RNA copies with incorporation of labeled nucleotides. For DNA, random priming or nick translation is used. Several alternative methods have also been developed.[11]

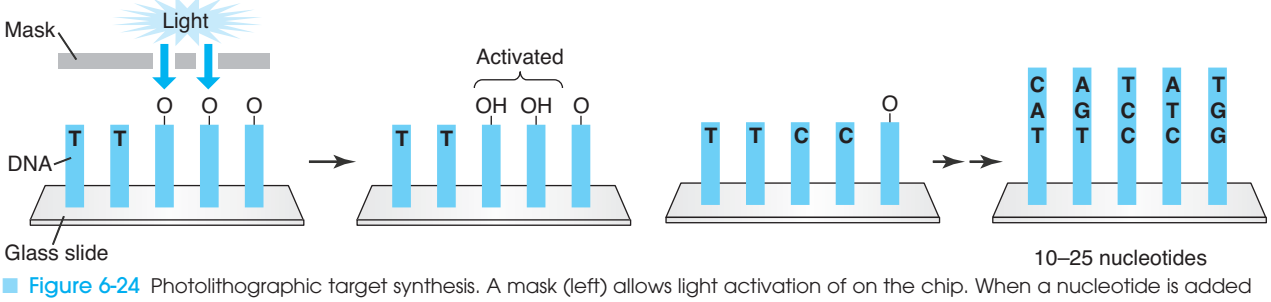

Figure 6-24 Photolithographic target synthesis. A mask (left) allows light activation of on the chip. When a nucleotide is added only the activated spots will covalently attach it (center). The process is repeated until the desired sequences are generated at each position on the chip (right).

For gene expression analyses, target probes immobilized on the chips are hybridized with labeled mRNA from treated cells or different cell types to assess the expression activity of the genes represented on the chip. Arrays used for this application are classifie as **expression arrays.**[12] Expression arrays measure transcript or protein production relative to a reference control isolated from untreated or normal specimens (Fig. 6-26).

Another application of array technology is **comparative genome hybridization** (array CGH). This method is used to screen the genome or specific genomic loci for deletions and amplifications.[13] For this method, genomic DNA is isolated, fragmented, and labeled for hybridization on the chip (Fig. 6-27). This type of method is analogous to the cytogenetic technique done on metaphase chromosomes. Array CGH can provide higher resolution and more defined genetic information than traditional cytogenetic analysis, but it is limited to the analysis of loci represented on the chip. Genomic arrays can be performed on fixed tissue and limiting samples. Methods have been developed to globally amplify genomic DNA to enhance CGH analysis.[14,15] Reading microarrays requires a fluorescent reader and analysis software. After determination of background and normalization with standards included on the array, the software averages the signal intensity from duplicate or triplicate sample data. The results are reported as a relative amount of the reference and test signals. Depending on the program, variances more than 2–3 standard deviations from 1 (test = reference) are considered an indication of significant increases (test:reference >1) or decreases (test:reference <1) in the test sample.

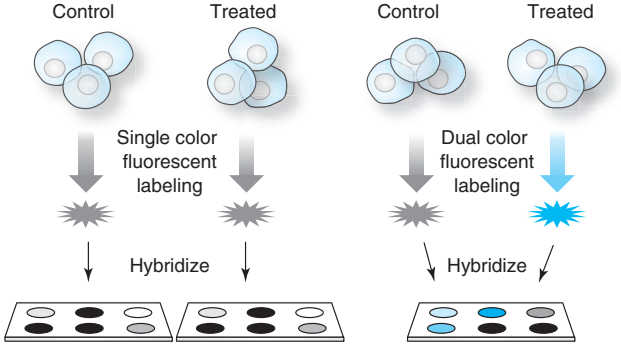

Figure 6-26 Labeling of sample for array analysis. At the left is single color fluorescent labeling where duplicate chips are hybridized separately and compared. On the right is dual color labeling where test (treated) and reference (control) samples are labeled with different color fluors and hybridized to the same chip.

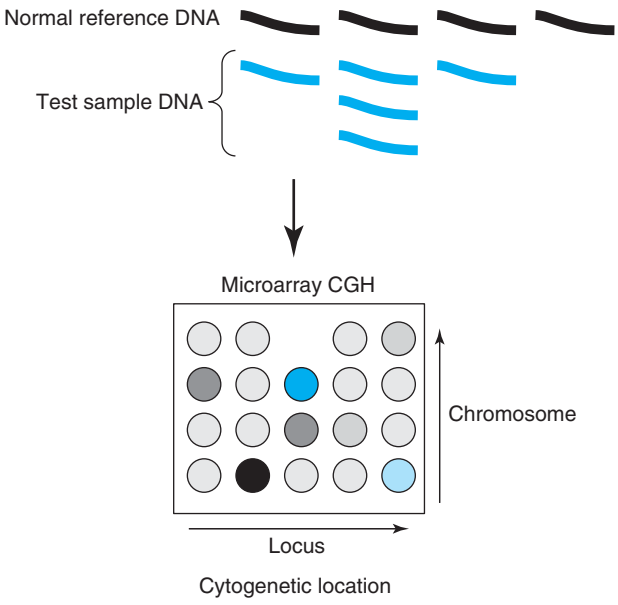

Figure 6-27 Comparative genomic hybridization. Reference and test DNA are labeled with different fluors, represented here as black and green respectively. After hybridization, excess green label indicates amplification of test sample locus. Excess black label indicates deletion of the test sample locus. Neutral or gray indicates equal test and reference DNA.

Several limitations to the array technology initially restrained the use of microarrays in the clinical laboratory. Lack of established standards and controls for optimal binding prevents the calibration of arrays from one laboratory to another. Not enough data have been accumulated to determine the background nonspecific binding and cross hybridization that might occur among and within a given set of sequences on an array. For instance, how much variation would result from comparing two normal samples together multiple times? Background "noise" can also affect the interpretation of array results. Furthermore, passive hybridization of thousands of different sequences will result in different binding affinities under the same stringency conditions, unless immobilized sequences are carefully designed to have similar melting temperatures. For mutation analysis, the length of the immobilized

probe is limited due to the use of a single hybridization condition for all sequences. For gene expression applications, only relative, rather than absolute, quantitation is possible.

These and other concerns are being addressed to improve the reliability and consistency of array analysis. As more data are accumulated, baseline measurements, universal standards, and recommended controls will be established. Advances in microelectronics and microfluidics have also been applied to array design and manufacture.[16,17]

Although arrays are, to date, in limited use in clinical laboratories, improvements in price and availability of instrumentation and premade chips increase their value for medical applications. Minimal sample requirements and comprehensive analysis with relatively small investments in time and labor are attractive features of array technology.

Solution Hybridization

Solution hybridization is not yet a routine part of clinical analysis. With the increasing interest in short interfering RNAs (siRNAs) and microRNAs (miRNAs), which are conveniently analyzed by this type of hybridization analysis, solution methods may come into more frequent use.

Solution hybridization has been used to measure mRNA expression, especially when there are low amounts of target RNA. One version of the method is called **RNase protection**, or S1 analysis, after the S1 single strand–specific nuclease. A labeled probe is hybridized to the target sample in solution. After digestion of excess probe by a single strand–specific nuclease, the resulting labeled, double-stranded fragments are resolved by polyacrylamide gel electrophoresis (Fig. 6-28). S1 mapping is useful for determining the start point or termination point of transcripts.[18,19] This procedure is more sensitive than Northern blotting because no target can be lost during electrophoresis and blotting. It is more applicable to expression analysis, the sensitivity being limited with double-stranded DNA targets.

There are several variations of this type of analysis. Probe:target hybrids can be detected by capture on a solid support or beads rather than by electrophoresis.[20,21] For these "sandwich"-type assays, two probes are used. Both hybridize to the target RNA. One probe, the capture probe, is biotinylated and will bind specifically to streptavidin immobilized on a plate or on magnetic beads. The other probe, called the detection probe, can be detected by a monoclonal antibody directed against RNA:DNA hybrids or a covalently attached digoxygenin molecule that can be used to generate chromogenic or chemiluminescent signal (see "Detection Systems").

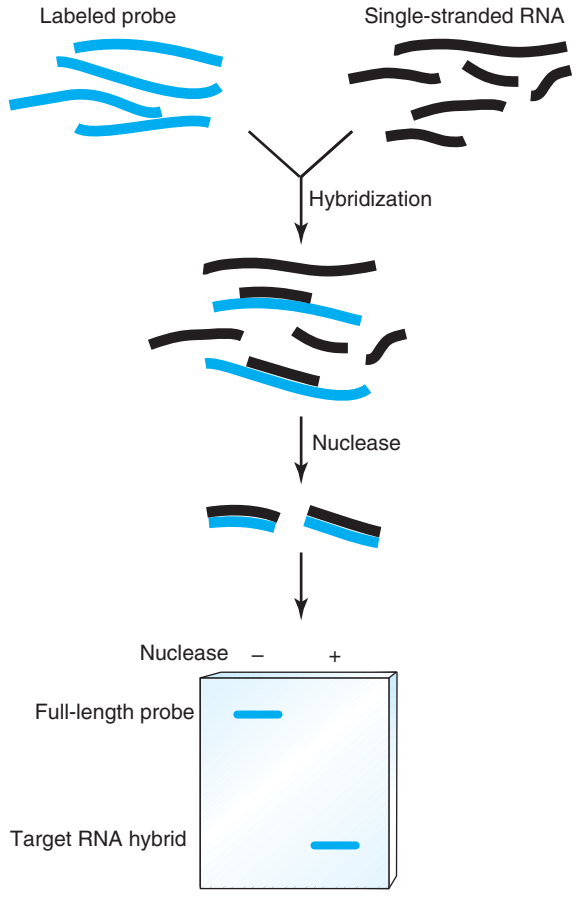

Figure 6-28 Solution hybridization. Target RNAs are hybridized to a labeled RNA or DNA carrying the complementary sequence to the target. After digestion by a single strand-specific nuclease, only the target:probe double-stranded hybrid remains. The hybrid can be visualized by the label on the probe after electrophoresis.

Solution hybridization can also be applied to the analysis of protein-protein interactions and to nucleic acid–binding proteins, using a **gel mobility shift**

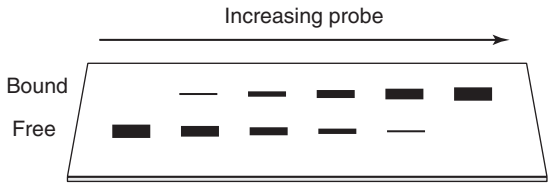

Figure 6-29 Gel mobility shift assay showing protein-protein or protein-DNA interaction. The labeled test substrate is mixed with the probe in solution and then analyzed on a polyacrylamide gel. If the test protein binds the probe protein or DNA, the protein will shift up in the gel assay.

assay.[22,23] After mixing the labeled DNA or protein with the test material, such as a cell lysate, a change in mobility, usually a shift to slower migration, indicates binding of a component in the test material to the probe protein or nucleic acid (Fig. 6-29). This assay has been used extensively to identify trans factors that bind to cis acting elements that control gene regulation.

Solution hybridization can also be used to detect sequence changes in DNA or mutational analysis. These applications will be discussed in Chapter 8.

Hybridization methods offer the advantage of direct analysis of nucleic acids at the sequence level, without cloning of target sequences. The significance of hybridization methodology to clinical applications is the direct discovery of molecular genetic information from routine specimen types. A wide variety of modifications of the basic blotting methods have and will be developed for clinical and research applications. Although amplification methods, specifically PCR, have replaced many blotting procedures, a number of hybridization methods are still used extensively in routine clinical analysis.

• STUDY QUESTIONS •

1. Calculate the melting temperature of the following DNA fragments using the sequences only:
   a. AGTCTGGGACGGCGCGGCAATCGCA
      TCAGACCCTG CCGCG CCGTTAGCGT
   b. TCAAAAATCGAATATTTGCTTATCTA
      AGTTTTTAGCTTATAAACGAATAGAT
   c. AGCTAAGCATCGAATTGGCCATCGTGTG
      TCGATTCGTAGCTTAACCGGTAGCACAC
   d. CATCGCGATCTGCAATTACGACGATAA
      GTAGCGCTAGACGTTAATGCTGCTATT

   Suppose you were to use single strands of these fragments as probes for a Southern blot:

2. If the fragments were dissolved in a solution of 50% formamide, is the stringency of hybridization higher or lower than if there were no formamide?

3. If a high concentration of NaCl were added to the hybridization solution, how would the stringency be affected?

4. Does heating of the solution from 65°C to 75°C during hybridization raise or lower stringency?

5. At the end of the procedure, what would the autoradiogram show if the stringency was too high?

6. In an array CGH experiment, three test samples were hybridized to three microarray chips. Each chip was spotted with eight gene probes (Gene A-H). Below are results of this assay expressed as the ratio of test DNA to reference DNA. Are any of the eight genes consistently deleted or amplified in the test samples? If so, which ones?

Gene	Sample 1	Sample 2	Sample 3
A	1.06	0.99	1.01
B	0.45	0.55	0.43
C	1.01	1.05	1.06
D	0.98	1.00	0.97
E	1.55	1.47	1.62
F	0.98	1.06	1.01
G	1.00	0.99	0.99
H	1.08	1.09	0.90

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Chapter 7

Lela Buckingham and Maribeth L. Flaws

Nucleic Acid Amplification

OUTLINE

OBJECTIVES

- Compare and contrast among the following in vitro assays for amplifying nucleic acids: polymerase chain reaction (PCR), branched DNA amplification, ligase chain reaction, transcription-mediated amplification, and Qβ replicase with regard to type of target nucleic acid, principle, major elements of the procedure, type of amplicon produced, major enzyme(s) employed, and applications.
- Describe examples of modifications that have been developed for PCR.
- Discuss how amplicons are detected for each of the amplification methods.
- Design forward and reverse primers for a PCR, given the target sequence.
- Differentiate between target amplification and signal amplification.

Early analyses of nucleic acids were limited by the availability of material to be analyzed. Generating enough copies of a single gene sequence required propagation of millions of cells in culture or isolation of large amounts of genomic DNA. If a gene had been cloned, many copies could be generated on bacterial plasmids, but this preparation was laborious, and some sequences were resistant to propagation in this manner.

The advent of the ability to amplify a specific DNA sequence opened the possibility to analyze at the nucleotide level virtually any piece of DNA in nature. The first specific amplification method of any type was the **polymerase chain reaction (PCR)**. Other amplification methods have been developed based on making modifications of PCR. The methods that have been developed to amplify nucleic acids can be divided into three groups, based on whether the target nucleic acid itself, a probe specific for the target sequence, or the signal used to detect the target nucleic acid is amplified. These methods are discussed in this chapter.

Target Amplification

The amplification of nucleic acids by **target amplification** involves making copies of a target sequence to such a level (in the millions of copies) that they can be detected in vitro. This is analogous to growing cells in culture and allowing the cells to replicate their nucleic acid as well as themselves so that, for example, they can be visualized on an agar plate. The difference is that waiting for cells to replicate to detectable levels can take days to weeks or months, whereas replicating the nucleic acid in vitro only takes hours to days. PCR is the first and prototypical method for amplifying target nucleic acid.

Polymerase Chain Reaction

Kary Mullis conceived the idea of amplifying DNA in vitro in 1983 while driving one night on a California highway.[1,2] In the process of working through a mutation detection method, Mullis came upon a way to double his test target, a short region of double-stranded DNA, giving him 2^1, or 2, copies. If he repeated the process, the target would double again, giving 2^2, or 4, copies. After N doublings, he would have 2^N copies of his target. If $N = 30$ or 40, there would be millions of copies.

What Mullis had envisioned was PCR. Over the next months in the laboratory, he synthesized oligos flanking a region of the human nerve growth factor and tried to amplify the region from human DNA, but the experiment did not work. Not sure of the nucleotide sequence information he had on the human genes, he tried a more defined target. The first successful amplification was a short fragment of the *Escherichia coli* plasmid, pBR322. The first paper describing a practical application, the amplification of beta-globin and analysis for diagnosis of patients with sickle cell anemia, was published 2 years later.[3] He called the method a "polymerase-catalyzed chain reaction" because DNA polymerase was the enzyme he used to drive the replication of DNA, and once it started the replication continued in a chain reaction. The name was quickly shortened to PCR. Since PCR was conceived and first performed, it has become increasingly user-friendly, more automated, and more amenable to use in a clinical laboratory, with infinite applications possible.

Basic PCR Procedure

When the cell replicates its DNA it requires the existing double-stranded DNA that serves as the **template** to give the order of the nucleotide bases, the deoxyribonucleotide bases themselves: adenine, thymine, cytosine, and guanine; DNA polymerase to catalyze the addition of nucleotides to the growing strand, and a primer to which DNA polymerase adds subsequent bases (refer to Chapter 1 for a detailed explanation).

PCR essentially duplicates the in vivo replication of DNA in vitro, using the same components (Table 7.1) to replicate DNA as the cell does in vivo, with the same end result, one copy of double-stranded DNA becoming two copies (Fig. 7-1). Within one to two hours PCR can produce millions of copies called **amplicons** of DNA. In contrast it would probably take days for a cell to produce the same number of copies in vivo. The real advantage of the PCR is the ability to amplify specific targets. Just as the Southern blot first allowed analysis of specific regions in a complex background, PCR presents the opportunity to amplify and essentially clone the target sequences. The amplified target, then, can be subjected to innumerable analytical procedures.

The components of the PCR, DNR template, primers, nucleotides, polymerase, and buffers, are subjected to an **amplification program.** The amplification program con-

Historical Highlights

Kary Mullis was working in a laboratory at Cetus Corporation, where he synthesized short single-stranded DNA molecules or oligodeoxynucleotides (oligos) used by other laboratories. Mullis also tinkered with the oligos he made. As he drove through the mountains of Northern California, Mullis was thinking about a method he had designed to detect mutations in DNA. His scheme was to add radioactive dideoxynucleotides, ddATP, ddCTP, ddGTP, ddTTP, to four separate DNA synthesis reactions containing oligos, template, and DNA polymerase. In each reaction, the oligo would bind specifically to the template, and the polymerase would extend the oligo with the dideoxynucleotide but only the dideoxynucleotide that was complementary to the next nucleotide in the template. He could then determine in which of the four tubes the oligo was extended with a radioactive ddNTP by gel electrophoresis. He thought he might improve the method by using a double-stranded template and priming synthesis on both strands, instead of one at a time. Because the results of the synthesis reaction would be affected by contaminating deoxynucleotides (dNTPs) in the reagent mix, Mullis considered running a preliminary reaction without the ddNTPs to use up any contaminating dNTPs. He would then heat the reaction to denature the dNTP-extended oligos and add an excess of unextended oligos and the ddNTPs. As he further considered the modification to his method, he realized that if the extension of an oligo in the preliminary reaction crossed the point where the other oligo bound on the opposite strand, he would make a new copy of the region between, similar to how a cell replicated its DNA during cell division. He considered the new copy and additional advantage, as it would improve the sensitivity of this method by doubling the target. Then he thought, what if he did it again? The target would double again. If he added dNTPs intentionally he could do it over and over again. In his own words: "I stopped the car at mile marker 46,7 on Highway 128. In the glove compartment I found some paper and a pen. I confirmed that two to the tenth power was about a thousand and that two to the twentieth power was about a million and that two to the thirtieth power was around a billion, close to the number of base pairs in the human genome. Once I had cycled this reaction thirty times I would be able to [copy] the sequence of a sample with an immense signal and almost no background."[82]

sists of a specified number of **cycles** that are divided into steps during which the samples are held at particular temperatures for designated times. The temperature will then determine the reaction that occurs, and changing the temperature changes the reaction. Table 7.2 shows the steps of a common three-step PCR cycle.

PCR starts with one double-stranded DNA target. In the first step (**denaturation**), the double-stranded DNA is denatured into two single strands in order to be replicated (Fig. 7-2). This is accomplished by heating the sample at 94°–96°C for several seconds to several minutes, depending on the template. The initial denaturation step is lengthened for genomic or other large DNA template fragments. Subsequent denaturations can be shorter.

The next and most critical step for the specificity of the PCR is the **annealing** step. In the second step of the PCR

Historical Highlights

Mullis' original method, using ddNTPs and oligos to detect mutations, is still in use today. **Fluorescent polarization-template-directed dye terminator incorporation** (described in Chapter 9) uses fluorescently labeled ddNTPs to distinguish which ddNTP extends the oligo. Another extension/termination assay, **Homogeneous MassExtend**, is a similar method, using mass spectrometry to analyze the extension products. Both of these methods are part of the Human Haplotype Mapping (HapMap) Project (see Chapter 11).

Historical Highlights

As Kary Mullis realized early on, the key to the brilliance of PCR is that primers can be designed to target specific sequences: "I drove on down the road. In about a mile it occurred to me that the oligonucleotides could be placed at some arbitrary distance from each other, not just flanking a base pair and that I could make an arbitrarily large number of copies of any sequence I chose and what's more, most of the copies after a few cycles would be the same size. That size would be up to me. They would look like restriction fragments on a gel. I stopped the car again. Dear Thor!, I exclaimed. I had solved the most annoying problems in DNA chemistry in a single lightening bolt. Abundance and distinction. With two oligonucleotides, DNA polymerase, and the four nucleoside triphosphates I could make as much of a DNA sequence as I wanted and I could make it on a fragment of a specific size that I could distinguish easily."[82]

cycle, the two oligonucleotides that will prime the synthesis of DNA anneal (hybridize) to complementary sequences on the template (Fig. 7-3). The primers dictate the part of the template that will be amplified; in other words, the primers determine the specificity of the amplification. It is important that the annealing temperature be optimized with the primers and reaction conditions. Annealing temperatures will range 50°–70°C and are usually established empirically. A starting point can be determined using the T_m of the primer sequences (see Chapter 6 for a discussion of stringency and hybridization). Reaction conditions, salt concentration, mismatches, template condition, and secondary structure will all affect the real T_m of the primers in the reaction.

Table 7.1 Components of a Typical PCR Reaction

Component	Purpose
0.25 mM each primer (oligodeoxynucleotides)	Directs DNA synthesis to the desired region
0.2 mM each dATP, dCTP, dGTP, dTTP	Building blocks that extend the primers
50 mM KCl	Monovalent cation (salt), for optimal hybridization of primers to template
10 mM Tris, pH 8.4	Buffer to maintain optimal pH for the enzyme reaction
1.5 mM $MgCl_2$	Divalent cation, required by the enzyme
2.5 units polymerase	The polymerase enzyme that extends the primers (adds dNTPs)
10^2–10^5 copies of template	Sample DNA that is being tested.

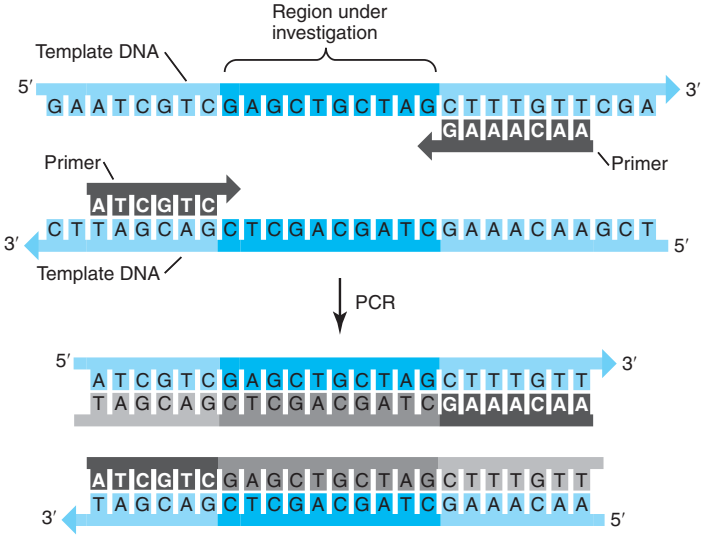

Figure 7-1 The components and result of a PCR. Oligodeoxynucleotides (primers) are designed to hybridize to sequences flanking the DNA region under investigation. The polymerase extends the primers making many copies of the region flanked by the primer sequences, the PCR product.

Table 7.2 **Elements of a PCR Cycle**

Step	Temperature (°C)	Time (sec)
Denaturation	90–96	20–60
Annealing	50–70	20–90
Extension	68–75	10–60

The third and last step of the PCR cycle is the primer extension step (Fig. 7-4). This is essentially when DNA synthesis occurs. In this step, the polymerase synthesizes a copy of the template DNA by adding nucleotides to the hybridized primers. DNA polymerase catalyzes the formation of the phosphodiester bond between an incoming dNTP determined by hydrogen bonding to the template (A:T or G:C) and the base at the 3′ end of the primer. In this way, DNA polymerase replicates the template DNA by simultaneously extending the **primers** on both strands of the template. This step occurs at the optimal temperature of the enzyme, 68°–72°C. In some cases, the annealing temperature is close enough to the extension temperature that the reaction can proceed with only two temperature changes. This is **two-step PCR**, as opposed to three-step PCR that requires a different temperature for all three steps.

At the end of the three steps, or one cycle (denaturation, primer annealing, and primer extension), one copy of double-stranded DNA has been replicated into two copies. Increasing the temperature back up to the denaturing temperature starts another cycle (Fig. 7-5), with the end result being a doubling in the number of double-stranded DNA molecules again (Fig. 7-6). At the end of the PCR program, millions of copies of the original region defined by the primer sequences will have been generated (Fig. 7-7). Following is a more detailed discussion of each of the components of PCR.

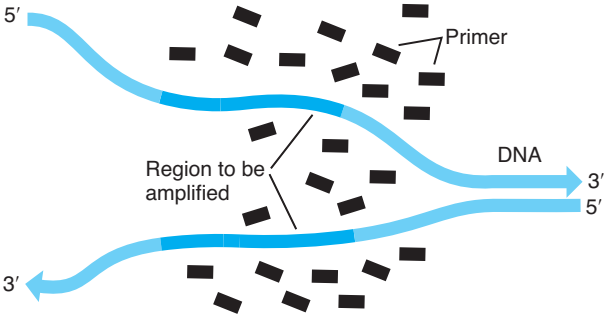

Figure 7-2 Denaturation of the DNA target. The region to be amplified is shown in green. The primers are present in vast excess.

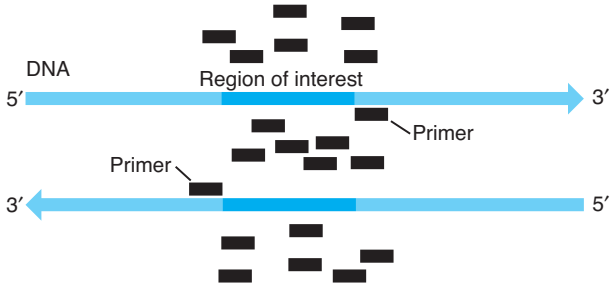

Figure 7-3 In the second step of the PCR cycle, annealing, the primers hybridize to their complementary sequences on each strand of the denatured template. The primers are designed to hybridize to the sequences flanking the region of interest.

Components of PCR

The PCR is a method of in vitro DNA synthesis. Therefore, to perform PCR, all of the components necessary for the replication of DNA in vivo are combined in optimal concentrations for replication of DNA to occur in vitro. This includes the template to be copied, primers to prime synthesis of the template, nucleotides, polymerase enzyme, and buffer components including monovalent and divalent cations to provide optimal conditions for accurate and efficient replication.

Figure 7-4 DNA polymerase catalyzes addition of deoxynucleotide triphosphates (dNTPs) to the primers, using the sample DNA as the template. This completes one PCR cycle. Note how in the original template there was one copy of the green region. Now, after one cycle, there are two copies.

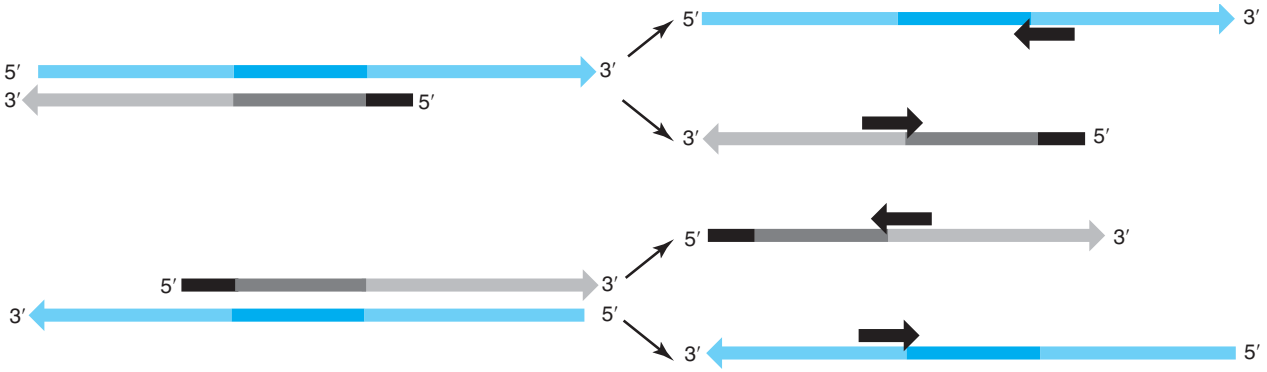

Figure 7-5 The first step (denaturation) of the second cycle, followed by the annealing step in which primers hybridize to the original template and the newly synthesized product.

Primers

The primers are the critical component of the PCR because primers determine the specificity of the PCR. Primers are analogous to the probes in blotting and hybridization procedures (see Chapter 6). Primers are chemically manufactured on a DNA synthesizer.

Primers are designed to contain sequences homologous to sites flanking the region to be analyzed. Primer design is therefore a critical aspect of the PCR. Primers are single-stranded DNA fragments, usually 20–30 bases in length. The forward primer must bind to the target DNA sequence just 5′ to the sequences intended to be amplified. The reverse primer must bind just 5′ to the sequence to be amplified on the opposite strand of the DNA. Thus, the design of primers requires some knowledge of the target sequence. The placement of the primers will also dictate the size of the amplified product.

Binding of primers is subject to the same physical limitations as probe binding. The primer sequence (% GC) and length affect the optimal conditions in which the primer will bind to its target. The approximate melting temperature, or T_m, of the primers can be calculated using the equation for short DNA fragments described in Chapter 6. The primer T_m can serve as a starting point for

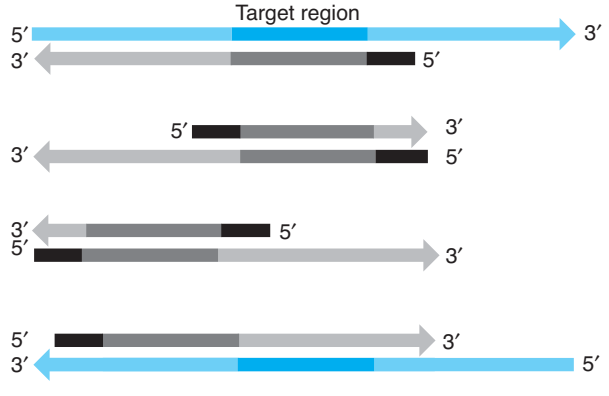

Figure 7-6 After the third step (extension) of the second cycle, there are four copies of the target region.

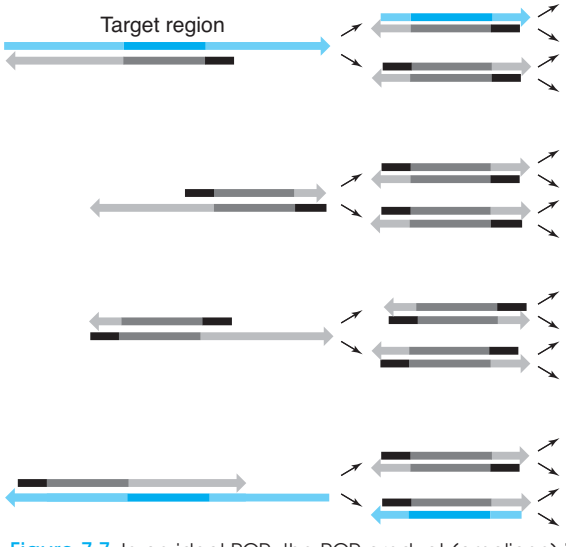

Figure 7-7 In an ideal PCR, the PCR product (amplicon) is composed of 2N copies of the target region where N = number of PCR cycles.

Advanced Concepts

For most laboratories, primers are purchased from a manufacturer by submitting the required sequences, amount required (scale of synthesis), and level of purification. Standard primer orders are on the 50–200–nm scale of synthesis. Higher amounts (1–50 μm) are more expensive to purchase per base. Purification of the synthesized primers may be performed by cartridge or column binding and washing, high performance liquid chromatography (HPLC), or by polyacrylamide gel electrophoresis (PAGE). HPLC and PAGE purification are more expensive than cartridge purification. Primers may also be labeled at the time of synthesis with fluorescent dyes, thiolation, biotinylization, or other modifiers.

setting the optimal annealing temperature, the critical step for the specificity of the amplification reaction. Primers should be designed such that the forward and reverse primers have similar T_m so that both will hybridize optimally at the same annealing temperature. T_m can be adjusted by increasing the length of the primers or by placing the primers in areas with more or fewer Gs and Cs in the template.

The nature of the primer structure determines the accuracy of binding to its complementary sequence and not to other sequences. Just as cross-hybridization can occur with blot hybridization, aberrant primer binding, or **mispriming**, can occur in PCR. A fragment synthesized from mispriming will carry the primer sequence and become a target for subsequent rounds of amplification (Fig. 7-8). Eventually, misprimed products will consume components away from the intended reaction. The resulting misprimed products may also interfere with proper interpretation of results or subsequent procedures such as sequencing (see Chapter 10) or mutation analysis (see Chapter 9).

Secondary structure (internal folding and hybridization within DNA strands) can also interfere with PCR. Primer sequences that have internal homologies, especially at the 3′ end, or homologies with the other member of the primer pair may not work as well in the PCR. An artifact often observed in the PCR is the occurrence of "**primer dimers**." These are PCR products that are just double the size of the primers. They result from the binding of primers onto each other through short (2–3 base) homologies at their 3′ ends and the copying of each primer sequence (Fig. 7-9). The resulting doublet is then a very efficient target for subsequent amplification.

The entire primer sequence does not have to bind to the template to prime synthesis; however, the 3′ nucleotide

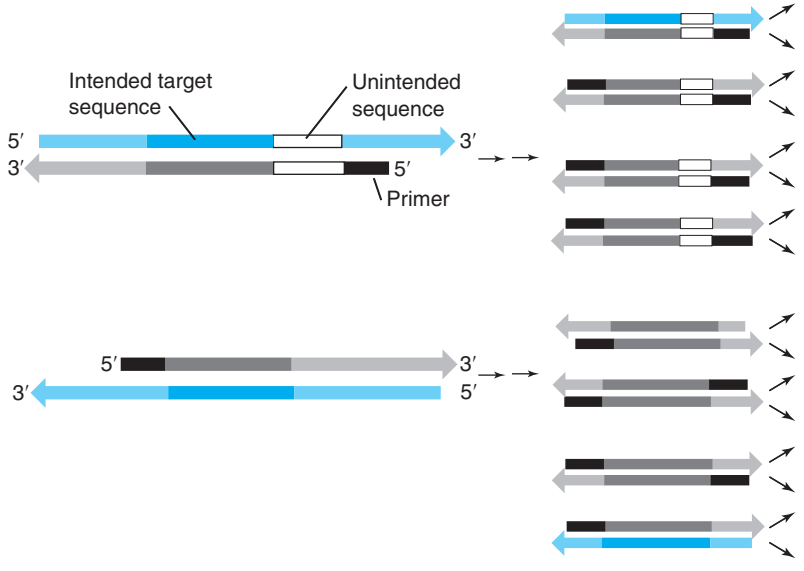

Figure 7-8 Mispriming of one primer creates an unintended product that could interfere with subsequent interpretation. Mispriming can also occur in regions unrelated to the intended target sequence.

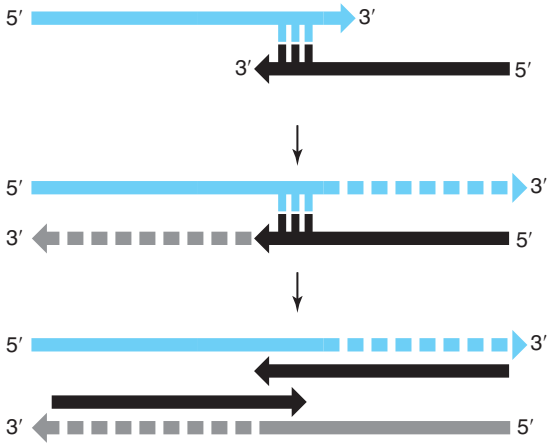

Figure 7-9 Formation of primer dimers occurs when there are three or more complementary bases at the 3′ end of the primers. With the primers in excess, these will hybridize during the annealing step (vertical lines), and the primers will be extended by the polymerase (dotted line) using the opposite primer as the template. The resulting product, denatured in the next cycle, will compete for primers with the intended template.

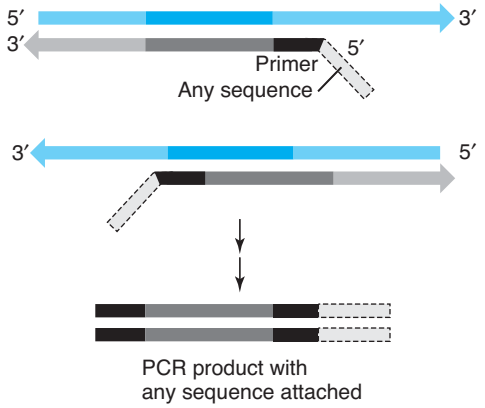

Figure 7-10 Any sequence can be added to the 5′ end of the primer. After PCR, the sequence will be on the end of the PCR product. These tailed primers can add useful sequences to one, as shown, or both ends of the PCR product.

position is critical for extension of the primer. The polymerase will not form a phosphodiester bond if the 3′ end of the primer is not hydrogen-bonded to the template. This characteristic of primer binding has been exploited to modify the PCR procedure for mutation analysis of the template (see Chapter 9). Noncomplementary extensions or tails can be added to the 5′ end of the primer sequences to introduce useful additions to the final PCR product, such as restriction enzyme sites, promoters, or binding sites for other primers. These **tailed primers** can be designed to add or alter sequences to one or both ends of the PCR product (Fig. 7-10).

DNA Template

The template may be single- or double-stranded DNA. In a clinical sample, depending on the application, the template may be derived from the patient's genomic or mitochondrial DNA or from viruses, bacteria, fungi, or parasites that might be infecting the patient. Genomic DNA will have only one or two copies per cell equivalent of single-copy genes to serve as amplification targets. With robust PCR reagents and conditions, nanogram amounts of genomic DNA are sufficient for consistent results. For routine clinical analysis, 100 ng to 1 μg of DNA is usually used. Lesser amounts are required for more defined template preparations such as cloned target DNA or product from a previous amplification.

The best templates are in good condition, free of contaminating proteins, and without nicks or breaks that can stop DNA synthesis or cause misincorporation of nucleotide bases. Templates with high GC content and secondary structure may prove more difficult to optimize for amplification. The DNA region affected in Fragile X syndrome, 5′ to the *FMR*-1 gene, is an example of such a GC-rich target.

Deoxyribonucleotide Bases

Nucleotide triphosphates are the building blocks of DNA. An equimolar mixture of the four deoxynucleotide-triphosphates (dNTPs), adenine, thymine, guanine, and

Advanced Concepts

Reagent systems that are designed to amplify targets optimally with high GC content are available. These systems incorporate an analog of dGTP, **deazaGTP**, to destabilize secondary structure. Deaza-GTP interferes with EtBr staining in gels and is best used in procedures with other types of detection, such as autoradiography.

cytosine, is added to the synthesis reaction in concentrations sufficient to support the exponential increase of copies of the template. The four dNTP concentrations should be higher than the estimated K_m of each dNTP (10–15 mM, the concentration of substrate at half maximal enzyme velocity). Standard procedures require 0.1–0.5 mM concentrations of each nucleotide. Substituted or labeled nucleotides, such as deaza GTP, may be included in the reaction for special applications. These nucleotides will require empirical optimization for best results.

DNA Polymerase

Automation of the PCR procedure was greatly facilitated by the discovery of the thermostable enzyme, *Taq* polymerase. When Kary Mullis first performed PCR, he used the DNA polymerase isolated from *E. coli*. Every time the sample was denatured, however, the high temperature denatured the enzyme. Thus, after each round of denaturation, additional *E. coli* DNA polymerase had to be added to the tube. This was labor-intensive and provided additional opportunities for the introduction of contaminants into the reaction tube. The *Taq* polymerase was isolated from the thermophilic bacterium, *Thermus aquaticus*.

Using an enzyme derived from a thermophilic bacterium meant that the DNA polymerase could be added once at the beginning of the procedure and it would maintain its activity throughout the heating and cooling cycles. Other enzymes, such as *Tth* polymerase from *Thermus thermophilus*, were subsequently exploited for laboratory use. *Tth* polymerase also has reverse transcriptase activity so that it can be used in reverse transcriptase PCR (**RT-PCR**, see below) where the starting material is an RNA template. The addition of proofreading enzymes, e.g., Vent polymerase allows *Taq* or *Tth* polymerase to generate large products over 30,000 bases in length.

Cloning of the genes coding for these polymerases has led to modified versions of the polymerase enzymes, such as the **Stoffel fragment** lacking the N-terminal 289 amino acids of *Taq* polymerase and its inherent 3′ to 5′ exonuclease activity[4]. The half-life of the Stoffel fragment at high temperatures is about twice that of *Taq* polymerase, and it has a broader range of optimal $MgCl_2$ concentrations (2–10 mM) than *Taq*. This enzyme is recommended for allele-specific PCR and for amplification of regions with high GC content. Further modified versions of the *Taq* enzymes retaining 3′ to 5′ exonuclease, but not 5′ to 3′ exonuclease activity, are used where high **fidelity** (accurate copying of the template) is important. Other variants of *Taq* polymerase, ThermoSequenase and T7 Sequenase, efficiently incorporate dideoxy NTPs for application to chain termination sequencing (see Chapter 10).

PCR Buffer

PCR buffers provide the optimal conditions for enzyme activity. Potassium chloride (20–100 mM), ammonium sulfate (15–30 mM), or other salts of monovalent cations are important buffer components. These salts affect the denaturing and annealing temperatures of the DNA and the enzyme activity. An increase in salt concentration makes longer DNA products denature more slowly than shorter DNA products during the amplification process, so shorter molecules will be amplified preferentially. The influence of buffer/salt conditions varies with different primers and templates.

Magnesium chloride also affects primer annealing and is very important for enzyme activity. Magnesium requirements will vary with each reaction, because each NTP will take up one magnesium atom. Furthermore, the presence of ethylenediaminetetraacetic acid (EDTA) or other chelators will lower the amount of magnesium available for the enzyme. Too few Mg^{2+} ions lower enzyme efficiency, resulting in a low yield of PCR product. Overly high Mg^{2+} concentrations promote misincorporation and thus increase the yield of nonspecific products. Lower Mg^{2+} concentrations are desirable when fidelity of the PCR is critical. The recommended range of $MgCl_2$ concentration is 1–4 mM, in standard reaction conditions. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mix-

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Note the nomenclature for the enzymes is derived from the organism from which the enzyme comes, similar to the nomenclature for restriction enzymes. For example, for the *Taq* polymerase, the "T" comes from the genus name, *Thermus,* and the *"aq"*comes from the species name, *aquaticus.*

ture should be adjusted accordingly. As with other PCR components, the optimal conditions are established empirically. Tris buffer and accessory buffer components are also important for optimal enzyme activity and accurate amplification of the intended product; 10 mM Tris-HCl maintains the proper pH of the buffer, usually between pH 8 and pH 9.5.

Accessory components are sometimes used to optimize reactions. Bovine serum albumin (10–100 μg/mL) binds inhibitors and stabilizes the enzyme. Dithiothreitol (0.01 mM) provides reducing conditions that may enhance enzyme activity. Formamide (1%–10%) added to the reaction mixture will lower the denaturing temperature of DNA with high secondary structure, thereby increasing the availability for primer binding. Chaotropic agents such as triton X-100, glycerol, and dimethyl sulfoxide added at concentrations of 1%–10% may also reduce secondary structure to allow polymerase extension through difficult areas. These agents contribute to the stability of the enzyme as well.

Enzymes are usually supplied with buffers optimized by the manufacturer. Commercial PCR buffer enhancers of proprietary composition may also be purchased to optimize difficult reactions. Often, the buffer and its ingredients are mixed with the nucleotide bases and stored as aliquots of a **master mix**. The enzyme, target, and primers are then added when necessary. Dedicated master mixes will also include the primers, so that only the target sequences must be added.

Thermal Cyclers

The first PCRs were performed using multiple water baths or heat blocks set at the required temperatures for each of the steps. The tubes were moved from one temperature to another by hand. In addition, before the discovery of thermostable enzymes, new enzyme had to be added after each denaturation step, further slowing the procedure and increasing the chance of error and contamination.

Automation of this tedious process was greatly facilitated by the availability of the heat stable enzymes. To accomplish the PCR, then, an instrument must only manage temperature according to a scheduled amplification program. **Thermal cyclers** or **thermocyclers** were thus designed to rapidly and automatically **ramp** (change) through the required incubation temperatures, holding at each one for designated periods.

Early versions of thermal cyclers were designed as heater/coolers with programmable memory to accept the appropriate reaction conditions. Compared with modern models, the available memory for recording the reaction conditions was limited, and sample capacity was small. Wax or oil (vapor barriers) had to be added to the reactions to prevent condensation of the sample on the tops of the tubes during the temperature changes. The layer of wax or oil made subsequent sample handling more difficult. Later, thermal cycler models were designed with heated lids that eliminated the requirement for vapor barriers.

There are numerous manufacturers of thermal cyclers. These instruments differ in heating and/or refrigeration systems as well as the programmable software within the units. Samples may be held in open chambers for air heating and cooling or in sample **blocks** designed to accommodate 0.2-mL tubes, usually in a 96 well format. Some models have interchangeable blocks to accommodate amplification in different sizes and numbers of tubes or slides. A cycler may run more than one block independently at the same time so that different PCR programs can be performed simultaneously. **Rapid PCR** systems are designed to work with very small sample volumes in chambers that can be heated and cooled quickly by changing the air temperature surrounding the samples. **Real-time PCR** systems are equipped with fluorescent detectors to measure PCR product as the reaction proceeds. PCR can also be performed in a microchip device in which 1–2 μL samples are forced through tiny channels etched in a glass chip, passing through temperature zones as the chip rests on a specially adapted heat block.[5]

For routine PCR in the laboratory, an appropriate amount of DNA that has been isolated from a test specimen is mixed with the other PCR components, either separately or as part of a master mix in 0.2–0.5–mL tubes. Most thermal cyclers take thin-walled tubes, 0.2-mL–tube strips or 96 well plates. Preparation of the specimen for PCR is often referred to as **pre-PCR** work. To avoid contamination (see below), it is recommended that the pre-PCR work be done in a designated area that is clean and free of amplified products. The sample tubes are then loaded into the thermal cycler. The computer is programmed with the temperatures and times for each step of the PCR cycle, the number of cycles to complete (usually 30–50), the conditions for ramping from step to

step, and the temperature at which to hold the tubes once all of the cycles are complete. The technologist starts the run and walks away until it is complete.

After the PCR, a variety of methods are used to analyze the PCR product. Most commonly, the PCR product is analyzed by gel or capillary electrophoresis. Depending on the application, the size, presence, or intensity of PCR products is observed on the gel. An example of the results from a PCR run is shown in Figure 7-11.

Controls for PCR

As with any diagnostic assay, running the correct controls in PCR is essential for maintaining and ensuring the accuracy of the assay. With every PCR run, the appropriate controls must be included. Positive controls ensure that the enzyme is active, the buffer is optimal, the primers are priming the right sequences, and the thermal cycler is cycling appropriately. A negative control without DNA (also called a **contamination control** or **reagent**

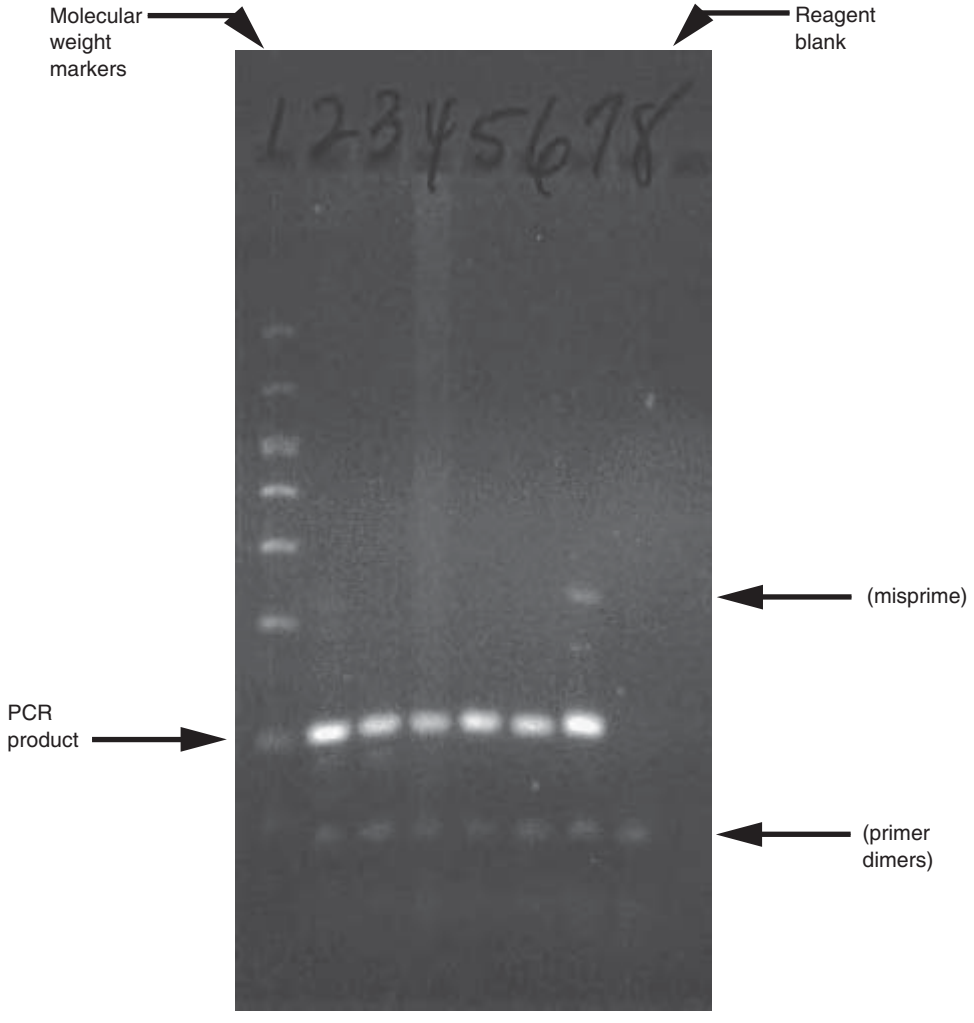

Figure 7-11 Example of PCR products after resolution on an agarose gel and staining with ethidium bromide. Molecular weight markers in lane 1 are used to estimate the size of the PCR product. The intended product is 100 bp. Artifactual primer dimers in every lane and a misprimed product in lane 7 can also be observed. Absence of products in lane 8 confirms that there is no contamination in the master mix.

blank) ensures that the reaction mix is not contaminated with template DNA or amplified products from a previous run. A negative control with DNA that lacks the target sequence (**negative template control**) ensures that the primers are not annealing to unintended sequences of DNA. In some applications of PCR, an internal control is included. In this type of control (**amplification control**), a second set of primers and an unrelated target are added to the reaction mix to demonstrate that the reaction is working even if the test sample is not amplified. Amplification controls are performed, preferably in the same tube with the test reaction, although it is acceptable to perform the amplification control on a duplicate sample. This type of control is most important when PCR results are reported as positive or negative, by which "negative" means that the target sequences are not present. The amplification control is critical to distinguish between a true negative for the sample and an amplification failure (false-negative).

Control of PCR Contamination

Contamination is a significant concern for methods that involve target amplification by PCR. The nature of the amplification procedure is such that, theoretically, a single molecule will give rise to product. This is critical in the clinical laboratory where results may be interpreted based on the presence, absence, size, or amount of a PCR product. With modern reagent systems designed for robust amplification of challenging specimens, such as paraffin embedded tissues or samples with low cell numbers, the balance between aggressive amplification of the intended target and avoidance of a contaminating template is delicate. For this reason, contamination control is of utmost importance in designing a PCR procedure and laboratory setup.

Although genomic DNA is a source of spurious PCR targets, the major cause of contamination is PCR products from previous amplifications. Unlike the relatively large and scarce genomic DNA, the small, highly concentrated PCR product DNA can aerosol when tubes are uncapped and when the DNA is pipetted. This PCR product is a perfect template for primer binding and amplification in a subsequent PCR using the same primers. Contamination control procedures, therefore, are mainly directed toward eliminating PCR product from the setup reaction.

Contamination is controlled both physically and chemically. Physically, the best way to avoid PCR carryover is to separate the pre-PCR areas from the post-PCR analysis areas. Positive airflow, air locks, and more extensive measures are taken by high throughput laboratories that process large numbers of samples and test for a limited number of amplification targets. Most laboratories can separate these areas by assigning separate rooms or using isolation cabinets. Equipment, including laboratory gowns and gloves, and reagents should be dedicated to either pre- or post-PCR. Items can flow from the pre- to the post-PCR area but not in the opposite direction without decontamination.

Ultraviolet (UV) light has been used to decontaminate and maintain pre-PCR areas. UV light catalyzes single- and double-strand breaks in the DNA that will then interfere with replication. Isolation cabinets are equipped with UV light sources that are turned on for about 20 minutes after the box has been used. The effectiveness of UV light may be increased by the addition of **psoralens** to amplification products after analysis. Psoralens intercalate between the bases of double-stranded DNA, and in the presence of long-wave UV light they covalently attach to the thymidines, uracils, and cytidines in the DNA chain. The bulky adducts of the psoralens prevent denaturation and amplification of the treated DNA.

The efficiency of UV light treatment for decontamination depends on the wavelength, energy, and distance of the light source. Care must be taken to avoid skin or eye exposure to UV light. UV light will also damage some plastics, so that laboratory equipment may be affected by extended exposure. Although convenient, the efficiency of UV treatment may not be the most effective decontaminant for every procedure.[6–8]

A widely used method for decontamination and preparation of the workspace is 10% bleach (7 mM sodium hypochlorite). Frequently wiping bench tops, hoods, or any surface that comes in contact with specimen material with dilute bleach or alcohol removes most DNA contamination. As a common practice in forensic work, before handling evidence or items that come in contact with evidence, gloves are wiped with bleach and allowed to air-dry.

Another widely used chemical method of contamination control is the dUTP-UNG system. This requires substitution of dTTP with dUTP in the PCR reagent master

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In addition to breaking the sugar phosphate backbone of DNA, UV light also stimulates covalent attachment of adjacent pyrimidines in the DNA chain, forming **pyrimidine dimers**. These boxy structures are the source of mutations in DNA in some diseases of sun exposure. DNA repair systems remove these structures in vivo. Loss of these repair systems is manifested in diseases such as xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy.[83] Psoralen in combination with UV light is an established treatment for psoriasis and other skin diseases.[84,85]

mix, which will result in incorporation of dUTP instead of dTTP into the PCR product. Although some polymerase enzymes may be more or less efficient in incorporation of the nucleotide, the dUTP does not affect the PCR product for most applications. At the beginning of each PCR, the enzyme uracil-N-glycosylase (UNG) is added to the reaction mix. This enzyme will degrade any nucleic acid containing uracil, such as contaminating PCR product from previous reactions. A short incubation period is added to the beginning of the PCR amplification program, usually at 50°C for 2–10 minutes to allow the UNG enzyme to function. The initial denaturation step in the PCR cycle will degrade the UNG before synthesis of the new products. Note that this system will not work with some types of PCR, such as nested PCR (discussed below), because a second round of amplification requires the presence of the first round product. The dUTP-UNG system is used routinely in real-time PCR procedures in which contamination control is more important because the contaminant will not be distinguishable from the desired amplicon by gel electrophoresis.

Prevention of Mispriming

As shown in Figure 7-11, PCR products are analyzed for size and purity by electrophoresis. The amplicon size should agree with the size determined by the primer placement. For instance, if two 20 b primers were designed to hybridize to sequences flanking a 100 bp target, the amplicon should be 140 bp in size. Much larger or smaller amplicons are due to mispriming or primer dimers or other artifacts of the reaction. For some procedures, these artifacts do not affect interpretation of results and, as long as they do not compromise the efficiency of the reaction, can be ignored. For other purposes, however, extraneous PCR products must be avoided or removed.

Misprimes are initially averted by good primer design and optimal amplification conditions. Even with the best conditions, however, misprimes can occur during preparation of the reaction mix. This is because *Taq* polymerase has some activity at room temperature. While mixes are prepared and transported to the thermal cycler, the primers and template are in contact at 22°–25°C, a condition of very low stringency (see Chapter 6 for a discussion of stringency). In these conditions, the primers can bind sequences other than their exact complements in the target. These misprimed products, then, are already present before the amplification program begins. Even using well-designed primers and optimizing amplification conditions, however, does not prevent all mispriming. To further prevent mispriming, **hot-start** PCR can be used.

Hot-start setup is done in three ways. In one approach, the reaction mixes are prepared on ice and placed in the thermal cycler after it has been prewarmed to the denaturation temperature. A second way to perform hot-start PCR is to use a wax barrier. A bead of wax is placed in the reaction tubes with all components of the reaction mix except enzyme and template. The tube is heated to 100°C to melt the wax and then cooled to room temperature. The melted wax will float to the top of the reaction mix in the tube and congeal into a physical barrier as it cools. The template and enzyme are then added on top of the wax barrier. When the tubes are placed in the thermal cycler, the wax will melt at the denaturation temperature, and the primers and template will first come in contact at the proper annealing temperature. The wax also serves as an evaporation barrier as the reaction proceeds. After amplification, however, the wax barrier must be punctured to gain access to the PCR products.

The third and most frequently used hot-start method is the use of sequestered enzymes, such as AmpliTaq Gold (Applied Biosystems), Platinum *Taq* (Invitrogen), JumpStart *Taq* (Sigma), and numerous others. These enzymes are either supplied in inactive form or the enzyme is inactivated by monoclonal antibodies or by other proprietary

methods. Regardless of the inactivation mechanism, the enzyme is inactive until it is activated by heat in the first denaturation step of the PCR program, preventing any primer extension during reagent mix preparation.

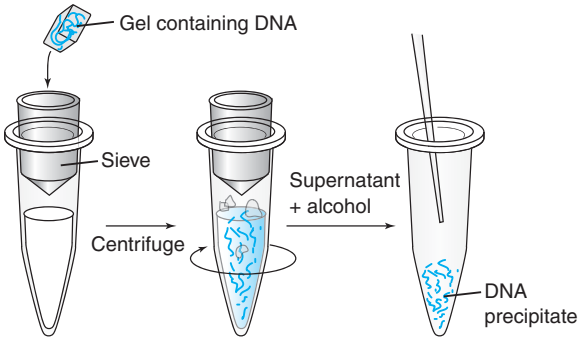
PCR Product Cleanup

Even the best procedures sometimes result in extraneous products. Sequence limitations to primer design or reaction conditions may not completely prevent primer dimers or misprimes. These unintended products are unacceptable for analytical procedures that demand pure product, such as sequencing or some mutation analyses (see Chapters 9 and 10). A direct way of obtaining clean PCR product is to resolve the amplification products by gel electrophoresis and then cut the desired bands from the gel and elute the PCR product. The gel slice can be digested with enzymes such as β-agarase (New England BioLabs) or iodine (Fig. 7-12). The agarase enzyme digests the agarose polymer and releases the DNA into solution for further purification.

Residual components of the reaction mix, such as leftover primers and unused nucleotides, also interfere with some post-PCR applications. Moreover, the buffers used for the PCR may not be compatible with post-PCR procedures. Amplicons free of PCR components are most frequently and conveniently prepared using spin columns (Fig. 7-13) or silica beads. The DNA binds to the column, and the rest of the reaction components are rinsed away by centrifugation. The DNA can then be eluted. Although columns or beads provide better recovery than gel elution, they may not completely remove residual primers.

Addition of shrimp alkaline phosphatase (SAP) in combination with exonuclease I (ExoI) is an enzymatic method for removing nucleotides and primers from PCR products prior to sequencing or mutational analyses. During a 15-minute incubation at 37°C, SAP dephosphorylates nucleotides, and ExoI degrades primers. The enzymes must then be removed by extraction or inactivated by heating at 80°C for 15 minutes. This method is convenient as it is performed in the same tube as the PCR. It does not, however, remove other buffer components.

In some post-PCR methods, such a small amount of PCR product is added to the next reaction that residual components of the amplification are of no consequence, so that no further clean up of the PCR product is required. The choice of clean-up procedure or whether clean up is necessary at all will depend on the application.



■ Figure 7-12 After gel electrophoresis, the gel band of PCR product is excised with a clean scalpel or spatula. The gel is disintegrated by centrifugation through a sieve, releasing the DNA. The DNA in solution can then be separated from the gel fragments, precipitated with alcohol and pelleted by a second centrifugation.

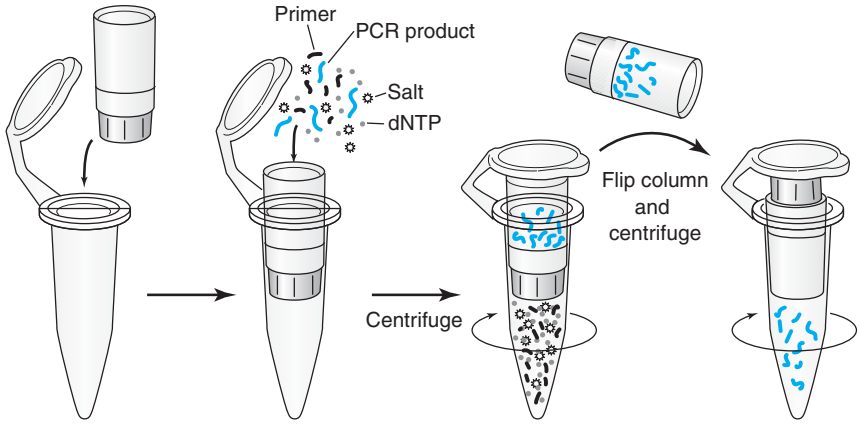
PCR Modifications

PCR today has been adapted for various applications. Several modifications are used in the clinical laboratory. Of the large (and increasing numbers) of PCR modifications, following is a description of those in standard use in the clinical molecular laboratory. These methods are capable of detecting multiple targets in a single run (multiplex PCR), using RNA templates (reverse transcriptase PCR), or such amplified products as templates (nested PCR) and quantitating starting template (quantitative PCR, or real-time PCR).

Multiplex PCR

More than one primer pair can be added to a PCR so that multiple amplifications are primed simultaneously, resulting in the formation of multiple products. **Multiplex PCR** is especially useful in typing or identification analyses. Individual organisms, from viruses to humans, can be identified or typed by observing a set of several PCR products at once. Pathogen typing and forensic identification kits contain multiple sets of primers that amplify polymorphic DNA regions. The pattern of product sizes will be specific for a given type or individual.

Multiple organisms have been the target of multiplex PCR in clinical microbiology laboratories.[9–11] One respiratory sample, for example, can be used to test for the presence of more than one respiratory virus.[12] Organisms



■ Figure 7-13 PCR product cleanup in spin columns (left) removes residual components in the PCR mix. Amplicon DNA binds to a silica matrix in the column while the buffer components flow through during centrifugation. The column is then inverted, and the DNA is eluted by another centrifugation in low salt (Tris-EDTA) buffer.

that cause sexually transmitted diseases can be targeted in multiplex PCR using one genital swab.[13] In a slightly different approach to testing for multiple targets, one set of primers can detect an infectious organism, and a second set can detect the presence of a gene that makes that organism resistant to a particular antimicrobial agent. This has been performed and published for methicillin-resistant *Staphylococcus aureus*.[14]

Multiplex PCR reagents and conditions require more complex optimization. Often, target sequences will not amplify with the same efficiency, and primers may interfere with other primers for binding to the target sequences. The conditions for the PCR must be adjusted for the optimal amplification of all products in the reaction. This may not be possible in all cases.

Multiplexing primers is useful, not only to detect multiple targets but also to confirm accurate detection of a single target. Internal amplification controls are often multiplexed with test reactions that are interpreted by the presence or absence of product. The control primers and targets must be chosen so that they do not interfere or compete with the amplification of the test region. Internal amplification controls are the ideal for positive/negative qualitative PCR tests.

Reverse Transcriptase PCR

Amplification by PCR requires a double-stranded DNA template. If the starting material for a procedure is RNA, it must first be converted to double-stranded DNA. This is accomplished through the action of reverse transcriptase (RT), an enzyme isolated from RNA viruses. This enzyme first copies the RNA single strand into a RNA:DNA hybrid strand and then uses a hairpin formation on the end of the newly synthesized DNA strand to prime synthesis of the homologous DNA strand, replacing the original RNA in the hybrid. The resulting double-stranded DNA is called **cDNA** for copy or complementary DNA. This product is adequate for PCR.

Like other DNA polymerases, reverse transcriptase requires priming. Specific primers, oligo dT primers or random hexamers, are most often used to prime the synthesis of the initial DNA strand. Specific primers will prime cDNA synthesis only from transcripts complementary to the primer sequences. The yield of cDNA will be relatively low using this approach but highly specific for the target of interest. Oligo dT primers are 18-b–long single-stranded polyT sequences that will prime cDNA synthesis only from messenger RNA with polyA tails. Yield of cDNA will be higher with oligo dT primers and should include all mRNA in the specimen. The highest yield of cDNA is achieved with random hexamers or decamers. These are 6–10–b–long single-stranded oligomers of random sequences. The 6–10–b sequences will match sequences in the target RNA with some frequency. Random priming will generate cDNA from all RNA (and DNA) in the specimen. For all strategies of cDNA preparation, the specificity of the final product is still determined by the PCR primers.

RT PCR is used to measure RNA expression profiles, to detect rRNA, to analyze gene regions interrupted by long introns, and to detect microorganisms with RNA genomes. For gene expression analysis, the amount of

cDNA reflects the amount of transcript in the preparation. In other applications, genes that are interrupted by long introns can be made more available for consistent amplification using cDNA versions lacking the interrupting sequences. cDNA is often used for sequencing because the sequence of the coding region can be determined without long stretches of introns complicating the analysis. The detection of RNA viruses such as *Coronavirus,* which is responsible for severe acute respiratory syndrome, can be accomplished using RT PCR.[15]

RT PCR was originally performed in two steps: cDNA synthesis and PCR. *Tth* DNA polymerase, which has RT activity and proprietary mixtures of RT and sequestered (hot-start) DNA polymerase, are components of one-step RT PCR procedures.[16] These methods are more convenient than the two-step procedure, as RNA is added directly to the PCR. The amplification program is modified to include an initial incubation of 45°–50°C for 30–60 minutes, during which RT makes cDNA from RNA in the sample. The RT activity will then be inactivated in the first denaturation step of the PCR procedure.

Although RT PCR is a widely used and important adjunct to molecular analysis, it is subject to the vulnerabilities of RNA degradation. As with other procedures that target RNA, specimen handling is important for accurate results. Methods have been described for the RT PCR amplification of challenging specimens, such as paraffin embedded tissues; however, fixed specimens are difficult to analyze consistently.[17]

Nested PCR

Increased sensitivity offered by the PCR is very useful in clinical applications as clinical specimens are often limited in quantity and quality. The low level of target and the presence of interfering sequences can prevent a regular PCR from working with the reliability required for clinical applications. **Nested PCR** is a modification that increases the sensitivity and specificity of the reaction.[18–21]

In nested PCR, two pairs of primers are used to amplify a single target in two separate PCR runs. The second pair of primers, designed to bind slightly inside of the binding sites of the first pair, will amplify the product of the first PCR in a second round of amplification. The second amplification will specifically increase the amount of the intended product. In **seminested PCR**, one of the second-round primers is the same as the first-round primer. Nested and seminested procedures increase specificity and sensitivity of the PCR (Fig. 7-14).

Several variations of nested and seminested PCR have been devised. For example, as shown in Figure 7-14, the first-round primers can have 5′ sequences added (5′ tails) complementary to sequences used for second-round primers. This tailed primer method is valuable for multi-

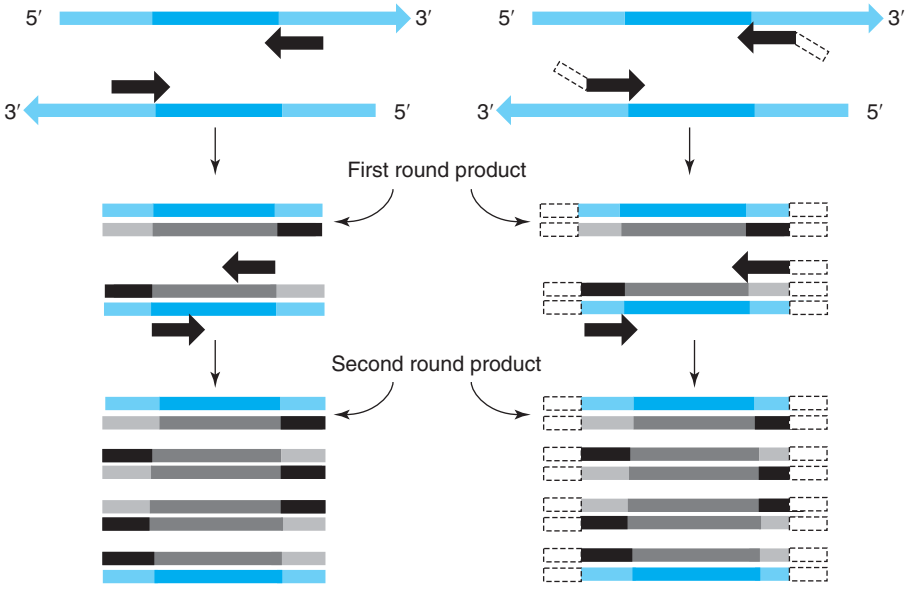


Figure 7-14 Variations of nested PCR using nested primers and seminested second-round primers (left) and tailed first-round primers (right).

plex procedures in which multiple first-round primers may differ in their binding efficiencies. Due to the tailed primers, sequences complementary to a single set of second-round primers are added to all of the first-round products. In the second round, then, all products will be amplified with the same primers and equal efficiency. Although this tailed primer procedure increases sensitivity in multiplex reactions, it does not increase specificity.

Real-Time (Quantitative) PCR

Standard PCR procedures will indicate if a particular target sequence is present in a clinical sample. For some situations, though, the clinician is also interested in how much of the target sequence is present. Several approaches have been taken to estimate the amount of starting template by PCR. By the nature of amplification, however, calculating direct quantities of starting material becomes complex. Strategies to quantitate starting material by quantitating the end products of PCRs have utilized internal controls, i.e., known quantities of starting material, that are co-amplified with the test template. These types of assays, however, suffer from primer incompatibilities and inconsistent results. Another approach is to add competitor templates at several known levels to assess the amount of test material by preferential amplification over a known amount of competitor.[22] These assays are also at times unreliable and inconsistent when test and internal control templates differ by more than 10-fold. They are most accurate with a 1:1 ratio of test and internal control, requiring analysis of multiple dilutions of controls for optimal results.

A very useful modification of the PCR process is **real-time** or **quantitative PCR** (**qPCR**).[23,24] This method was initially performed by adding ethidium bromide (EtBr) to a regular PCR. Because EtBr intercalates into double-stranded DNA and fluoresces, it can be used to monitor the accumulation of PCR products during the PCR in real time, i.e., as it is made. The advantage of this method over standard PCR is the ability to determine the amount of starting template accurately. These quantitative measurements are performed with the ease and rapidity of standard PCR without tedious addition of competitor templates or multiple internal controls. A growing number of clinically significant parameters, such as copy numbers of diseased human genes, viral load, tumor load, and the effects of treatment, are measured easily with this method.[25–27]

The rationale of qPCR is illustrated in Figure 7-15. If the target copy number in a PCR were graphed versus the number of cycles, the results would be an exponential curve where the number of target copies = 2^N, N being the number of cycles. If the copy number is measured by detectable fluorescence as shown in the figure, the curve looks similar to a bacterial growth curve, with a lag phase, an exponential (log) phase, a linear phase, and a stationary phase.

In contrast to real-time PCR, analysis of PCR product by the standard method occurs at the end of the PCR stationary phase (**endpoint analysis**). Exhaustion of reaction components and competition between PCR product and primers during the annealing step slow the PCR product accumulation after the exponential phase of growth until it finally plateaus. In the endpoint analysis, products of widely different starting template amounts are tested at the plateau where they are all the same (observe the ends of the amplification curves shown in Figure 7-15A.). Using the fluorescent signal to detect the growing target copy number during the amplification process, analysis in real-time PCR is performed in the exponential phase of growth where the accumulation of fluorescence is inversely proportional to the amount of starting template. With 10-fold dilutions of known positive standards, a relationship between the starting target copy number and the cycle number at which fluorescence crosses a threshold amount of fluorescence can be established.

The PCR cycle at which sample fluorescence crosses the threshold is the **threshold cycle**, or $\mathbf{C_T}$. Plotting the target copy number of the diluted standards against C_T for each standard generates the graph shown in Figure 7-15B. Once this relationship is established, the starting amount of an unknown specimen can be determined by the cycle number at which the unknown crosses the fluorescence threshold.

Advanced Concepts

The optimal threshold level is based on the background or baseline fluorescence and the peak fluorescence in the reaction. Instrument software is designed to set this level automatically. Alternatively, the threshold may be determined and set manually.

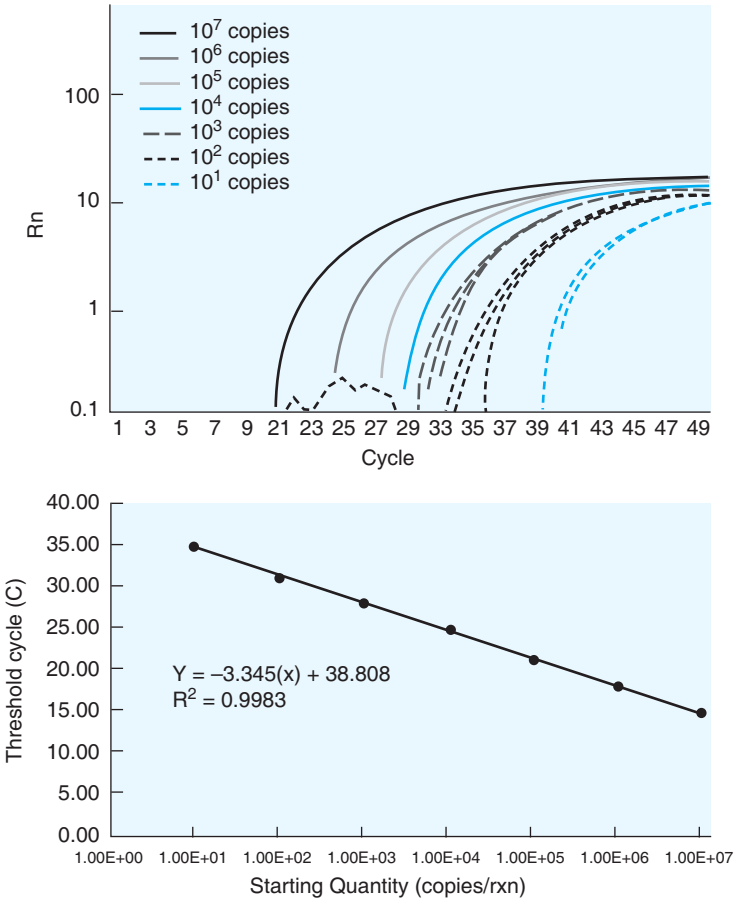


Figure 7-15 A plot of the accumulation of PCR product over 50 cycles of PCR (A) is a sigmoid curve. The generation of fluorescence occurs earlier with more starting template (solid lines) than with less (dotted lines). The cycle number at which fluorescence increases over a set amount, or fluorescence threshold, is inversely proportional to the amount of starting material.

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Fluorescence vs. C_T is an inverse relationship. The more starting material, the fewer cycles are necessary to reach the fluorescence threshold. Samples that differ by a factor of 2 in the original concentration of target are expected to be 1 cycle apart, with the more dilute sample having a C_T 1 cycle higher than the more concentrated sample. Samples that differ by a factor of 10 (as in a 10-fold dilution series) would be ~3.3 cycles apart. The slope of a standard curve made with 10-fold dilutions, therefore, should be –3.3.

The first approach to real-time PCR utilized the double-stranded DNA-specific dye EtBr. This method is still used for routine qPCR, except that EtBr has been replaced by SYBR green, another double-stranded DNA-specific dye. SYBR green is preferred for routine procedures because it has the specificity and robust fluorescence of EtBr but is less toxic than EtBr. These dyes bind and fluoresce specifically in the double-stranded DNA product of the PCR (Fig. 7-16).

The use of nonspecific dyes to measure product accumulation requires a clean PCR free of misprimed products or primer dimers, because these artifactual products will also generate fluorescence. More specific systems, examples of which are described below, have been devised that utilize probes designed to generate fluores-

Advanced Concepts

EtBR is a planar molecule that intercalates between the planar nucleotides in the DNA molecule. In doing so, it interferes with DNA metabolism and replication in vivo and is a mutagen. In contrast, SYBR green binds to the minor groove of the double helix without disturbing the nucleotide bases and thus does not upset DNA metabolism to the extent that EtBr does.

Advanced Concepts

The 5′ end of a Taqman probe is labeled with one of a number of dyes with different "colors," or peak wavelengths of fluorescence, e.g., FAM (6-carboxy-fluorescein), TET (6-tetrachlorofluorescein), HEX (6-hexachlorofluorescein), JOE (4′,5′-dichloro-2′,7′-dimethoxy-fluorescein), Cy3, Cy5 (indodicarbocyanine), and so forth. The probe is covalently bound at the 3′ end with a quencher, such as DABCYL (4-dimethylaminophenylazobenzoic acid) or TAMRA (5(6)-carboxytetramethylrhodamine), or nonfluorescent quenchers, such as BHQ1, BHQ2 (Black Hole Quenchers), and Eclipse. In the TaqMan system, the quencher prevents fluorescence from the 5′ dye until they are separated during the synthesis reaction.

cence. The probes increase specificity by only yielding fluorescence when they hybridize to the target sequences.

TaqMan was developed from one of the first probe-based systems for real-time PCR.[28] This method exploits the natural 5′ to 3′ exonuclease activity of *Taq* polymerase to generate signal. The original method reported by Holland et al. used radioactively labeled probe and measured activity by the release of radioactive cleavage fragments. The TaqMan procedure measures fluorescent signal generated by separation of dye and quencher, a system developed by Lee et al.[29] Here, a probe composed of a single-stranded DNA oligomer homologous to a specific sequence in the targeted region of the PCR template is used. Note that this probe is present in the PCR in addition to the specific primers used to prime the DNA synthesis reaction. The probe is chemically modified at its 3′ end so that it cannot be extended by the polymerase. The single-stranded DNA TaqMan probe is covalently attached to a fluorescent dye on one end and another dye or nonfluorescent molecule that pulls fluorescent energy from the 5′ dye (**quencher**) on the other (Fig. 7-17).

As the polymerase proceeds to synthesize DNA from the template to which the probe is hybridized, the natural exonuclease activity of *Taq* polymerase will degrade the probe into single and oligonucleotides, thereby removing the labeled nucleotide from the vicinity of the quencher and allowing it to fluoresce (Fig. 7-18). Excess probe is present so that with every doubling of the target sequences more probe binds and is digested, and more fluorescence is generated. The TaqMan procedure has been applied to quantitative determinations in oncology[30] and

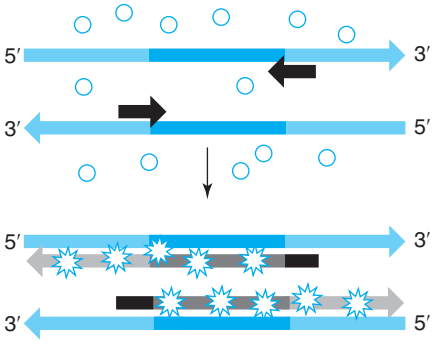

Figure 7-16 Non-sequence–specific dyes such as EtBr and SYBR green bind to double-stranded DNA products of the PCR. As more copies of the target sequence accumulate, the fluorescence increases.

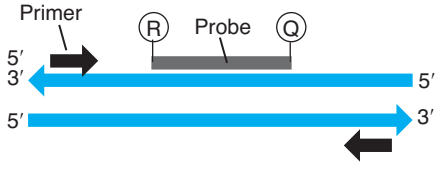

Figure 7-17 A TaqMan probe hybridizes to the target sequences between the primer binding sites. The probe is covalently attached to a fluorescent reporter dye (R) at the 5′ end and a quencher (Q) at the 3′ end.

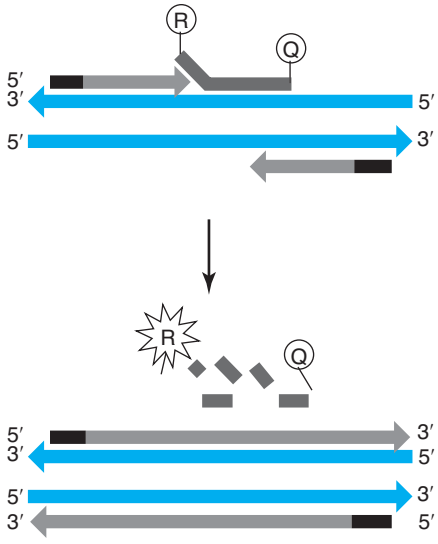

Figure 7-18 Taqman signal fluorescence is generated when *Taq* polymerase extends the primers and digests the probe and releases the reported from the vicinity of the quencher.

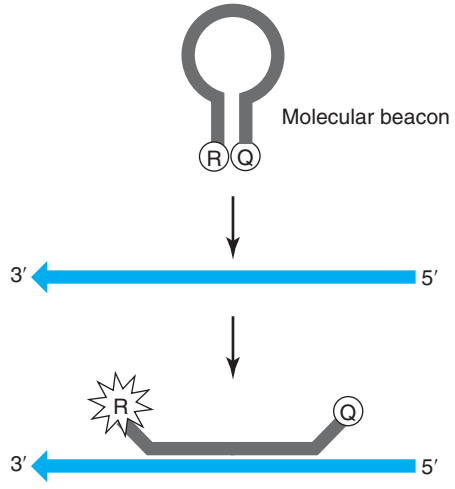

Figure 7-19 A molecular beacon probe contains target specific sequences and a short inverted repeat that hybridizes into a hairpin structure. The 5′ end of the probe has a reporter dye (R), and the 3′ end has a quencher dye (Q).

microbiology.[31] Probe design, like primer design, is important for a successful qPCR amplification.[32,33]

Another probe-based detection system, **Molecular Beacons**, measures the accumulation of product at the annealing step in the PCR cycle. The signal from Molecular Beacons is detectable only when the probes are bound to the template *before* displacement by the polymerase. Here the probe is chemically modified so that it is not degraded during the extension step. Molecular beacons are designed with a ~25-b–specific binding sequence flanked by a short (~5 b) inverted repeat that will form a stem and loop structure when the probe is not bound to the template. There is a reporter fluorophor (dye) at the 5′ end of the oligomer and quencher at the 3′ end. Until specific product is present, the probe will form a hairpin structure that brings the fluorophore in proximity with the quencher (Fig. 7-19). Fluorescence will occur on binding of the probe to denatured template during the annealing step (Fig. 7-20). When the primers are extended in the PCR, displacement of the probe by *Taq* will restore the hairpin (nonfluorescent) structure. Excess probe in the reaction mix will assure binding to the increasing amount of target. The amount of fluorescence, therefore, will be directly proportional to the amount of template available for binding and inversely proportional to the C_T.

Scorpion-type primers are a variation of Molecular Beacons.[34,35] In contrast to free-labeled probes, the PCR product will be covalently bound to the dye. In this system, target-specific primers are tailed at the 5′ end with a sequence homologous to part of the internal primer sequence, a quencher, a stem-loop structure, and a 5′ fluorophore (Fig. 7-21). The fluor and the quencher are positioned so that they are juxtaposed when the hairpin in the primer is intact. After polymerization, the secondary structure of the primer is overcome by hybridization of the primer sequence with the target sequence, removing the fluor from the quencher. This intramolecular system generates signal faster than the intermolecular Molecular Beacon strategy and may be preferred for methods requiring fast cycling conditions.[36]

Another frequently used system, **fluorescent resonance energy transfer** (**FRET**), utilizes two specific probes, one with a 3′ fluorophore (acceptor), the other with a 5′ catalyst for the fluorescence (donor) that binds to adjacent targets.[37] Examples of frequently used donor-acceptor pairs are fluorescein-rhodamine, fluorescein-(2 aminopurine), and fluorescein-Cy5. When the donor and acceptor are brought within 1–10 nm (1–5 bases) through specific DNA binding, excitation energy is transferred from the donor to the acceptor (Fig. 7-22). The acceptor

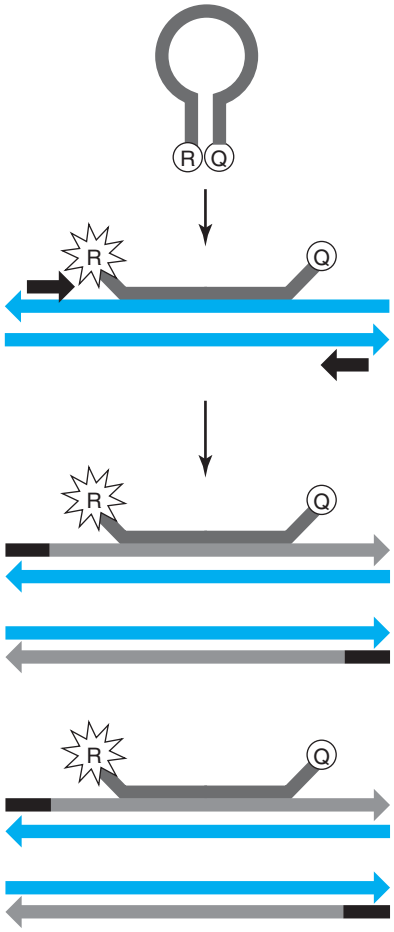

Figure 7-20 Molecular beacons bound to target sequences fluoresce. Fluorescence doubles with every doubling of target sequences.

then loses the energy in the form of heat or fluorescence emission called sensitized emission. The sequences of FRET probes are designed such that they bind 1–5 bases from each other on the target sequences. When they are bound to the target, the fluorescence is catalyzed. As with the molecular beacons, the more template available for binding, the more fluorescence will be generated. FRET probes are used frequently in the clinical laboratory for viral detection and quantitation and for amplification and detection of genetic diseases.[38–41]

Real-time PCR lends itself to several variations of technique as exemplified above. These techniques can be further modified, e.g., using FRET probes with different

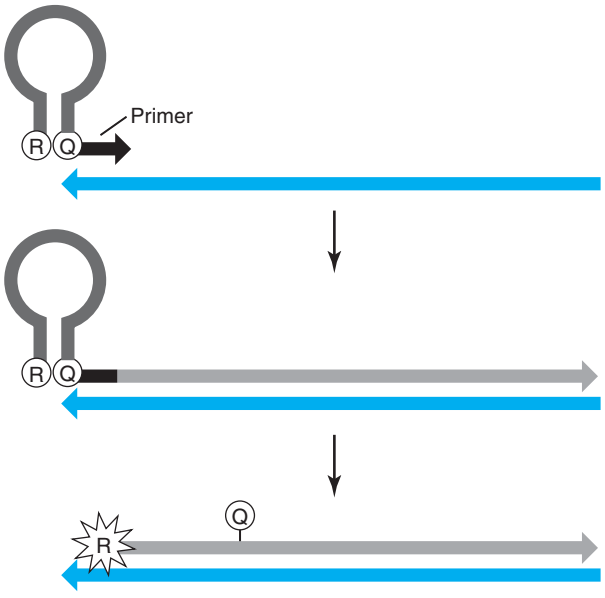

Figure 7-21 Scorpion primer/probes are primers tailed with molecular beacon-type sequences. After extension of the primer/probe, the target-specific sequences fold over to hybridize with the newly synthesized target sequences, separating the reporter (R) from the quencher (P).

sequences to distinguish types of organisms or to detect mutations. Refer to Chapter 9 for a description of methods using fluorescent probes and melt curves to detect gene mutations. Methods can be combined, e.g., using an intercalating dye (acridine orange) as the donor and a probe with a single receptor dye (rhodamine).[42] As with standard PCR, large and growing numbers of such methods have been devised for a variety of applications.

Arbitrarily Primed PCR

In arbitrarily primed PCR, also known as **randomly amplified polymorphic DNA** or random amplification of polymorphic DNA (RAPD), short (10–15 bases) primers with random sequences are used to amplify arbitrary regions in genomic DNA under low stringency conditions.[43,44] With this method, PCR products are generated without knowing the sequence of the target or targeting a specific gene. It is possible with this method to obtain multiple products, depending how many times a short sequence appears in the genome; in traditional PCR, only one product is generally obtained (Fig. 7-23). Arbitrarily

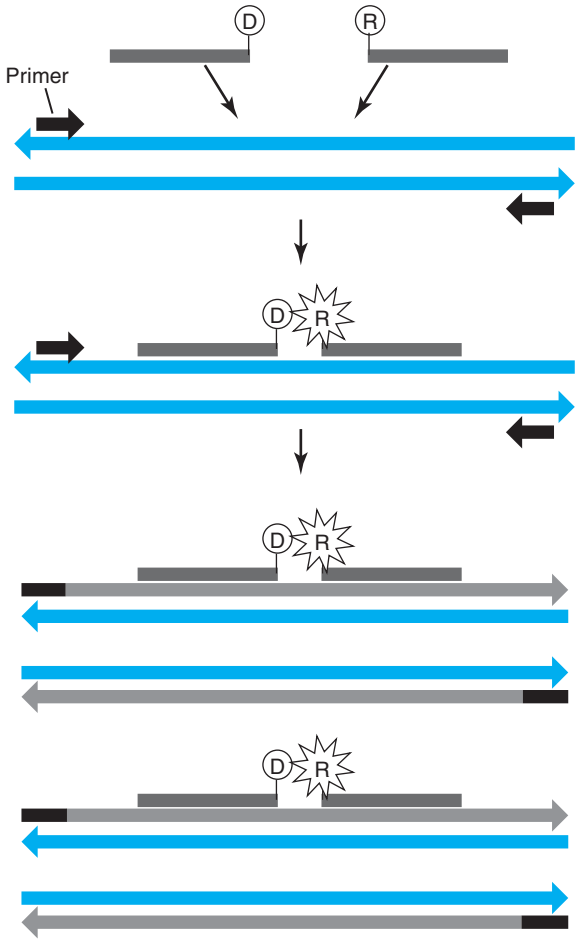

Figure 7-22 FRET probes are separate oligomers, one covalently attached to a donor fluor (D) and one to an acceptor or reporter fluor (R). The acceptor/reporter will fluoresce only when both probes are bound next to one another on the target sequences. As more target accumulates, more probes bind, and more fluorescence is emitted.

primed PCR has been used primarily in the epidemiological typing of microorganisms.[45] Similar band patterns obtained from performing PCR with the same arbitrary primers indicate that two organisms are the same or similar. The disadvantage of this method, though, is that the stringency is low enough that the reproducibility between runs is not very good, such that two organisms that had the same PCR product pattern on one day could have two different patterns and look like two different organisms when amplified on another day.

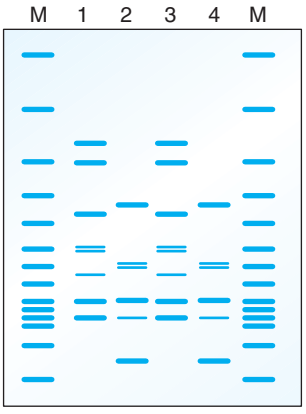

Figure 7-23 Illustration of results from a RAPD PCR. The first lane on the left contains molecular weight markers. Strain differences are evident from the different band patterns. Lanes 1 and 3 are the same strain, and lanes 2 and 4 are the same strain, but different from that in lanes 1 and 3. Lanes M, molecular weight markers

Transcription-Based Amplification Systems

In transcription-based amplification systems (TAS), RNA is the usual target instead of DNA. A DNA copy is synthesized from the target RNA, and then transcription of the DNA produces millions of copies of RNA. There are a number of commercial variations of this process: **transcription-mediated amplification** (TMA) (Gen-Probe), **nucleic acid sequence–based amplification** (NASBA) (Organon-Teknika), and **self-sustaining sequence replication** (3SR) (Baxter Diagnostics).

Kwoh and colleagues developed the first TAS in 1989.[46] TAS differs from other nucleic acid amplification procedures in that RNA is the target as well as the primary product. In the original method of TAS, a primer complementary to sequences in the target RNA that also has the binding site for RNA polymerase at one end is added to a sample of target RNA. The primer anneals, and reverse transcriptase makes a DNA copy of the target RNA. Heat is used to denature the DNA/RNA hybrid, and a second primer binds to the cDNA and is extended by reverse transcriptase producing double-stranded DNA. RNA polymerase derived from the bacteriophage T7 then transcribes the cDNA, producing hundreds to thousands of copies of RNA. The transcribed RNA can then serve as target RNA to which the primers bind and synthesize more cDNA.

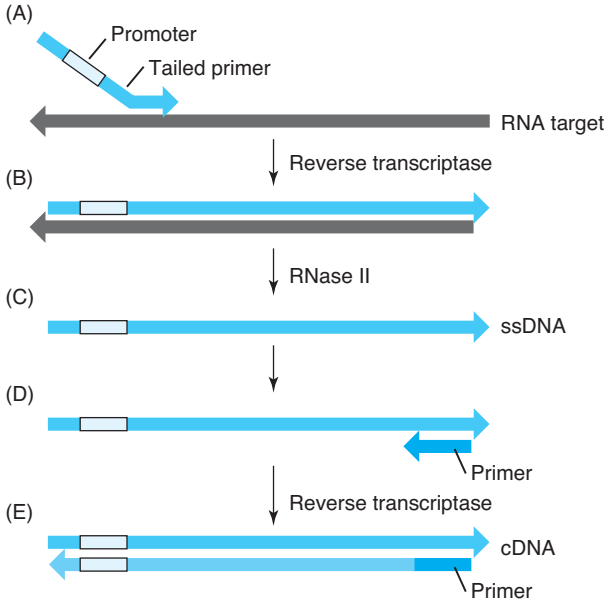

Figure 7-24 The first step in transcription-based amplification is the production of a complementary double-stranded DNA copy of the RNA target (A). Synthesis is performed by reverse transcriptase, which extends a primer that is tailed with an RNA polymerase binding site (promoter) sequence (green). The RNA/DNA hybrid (B) is digested with RNase H, leaving the single-stranded DNA (C), which is converted to a double strand with a complementary primer (D). The DNA product will have a promoter sequence at one end.

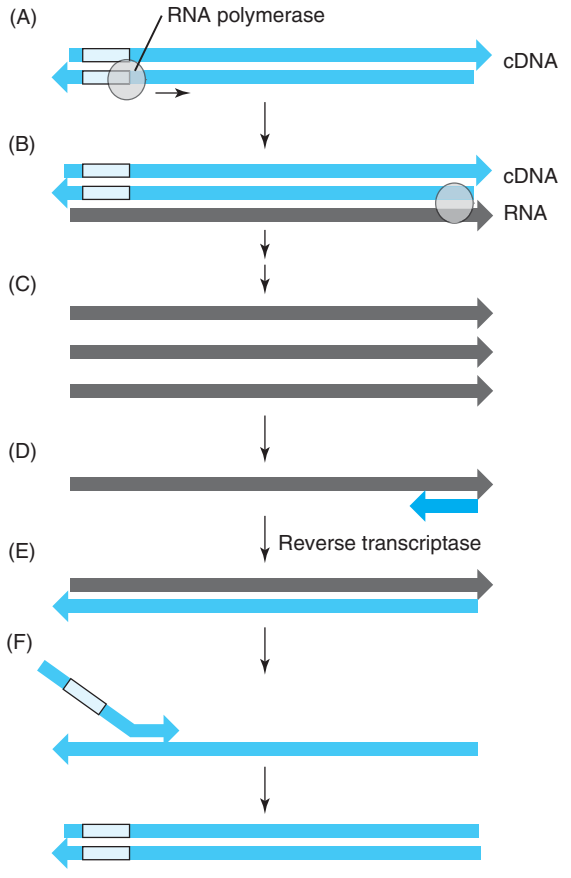

Figure 7-25 The cDNA produced by reverse transcriptase serves as a template for RNA polymerase (A). Many copies of RNA are synthesized (B), (C) which are primed by a complementary primer (D) for synthesis of another RNA/DNA hybrid (E). After RNase H degrades the RNA strand, the primer tailed with the promoter sequences synthesizes another template (F), cycling back into the system as (A).

The original TAS procedure as described above had the disadvantage that heat denaturation was required to denature the intermediate RNA/DNA hybrid product. The heat also denatured the enzymes so that fresh enzyme had to be added after each denaturation step. The process was simplified with the addition of RNase H derived from *E. coli* (Fig. 7-24). RNase H degrades the RNA from the intermediate hybrid, eliminating the heating step. Thus, after synthesis of the DNA copy by reverse transcriptase, the RNA strand is degraded by RNase H. Binding of the second primer and extension of the primer producing double-stranded DNA by reverse transcriptase is followed by transcription of the cDNA with T7 RNA polymerase (Fig. 7-25).

This modified procedure has been marketed as 3SR, NASBA, or TMA, depending on the manufacturer. An additional modification and simplification of the procedure came about with the discovery that the reverse transcriptase derived from avian myeloblastosis virus (AMV) has inherent RNase H activity. Thus, TAS can be run with only two enzymes, AMV reverse transcriptase and T7 RNA polymerase.

TAS has some advantages over PCR and other amplification procedures. First, in contrast to PCR and the ligase chain reaction (LCR, discussed below), TAS is an isothermal process, negating the requirement for thermal cycling to drive the reactions. Second, targeting RNA allows for the direct detection of RNA viruses, e.g., Hepatitis C Virus[47,48] and Human Immunodeficiency Virus.[49,50] Even targeting the RNA of other organisms, such as *Mycobac-*

terium tuberculosis, is more sensitive than targeting the DNA, because each bacterium, for example, has multiple copies of RNA, whereas it has only one copy of DNA.[51]

The NASBA procedure with slight modifications can also be performed on a DNA target.[52] For DNA, the sample is heated to denature the DNA, and the first primer anneals and is extended by reverse transcriptase (which in addition to having RNA-dependent DNA polymerase activity also has DNA-dependent DNA polymerase activity). The sample is heated again to denature the double strands, and the second primer binds and is extended. The DNA product has also incorporated the T7 RNA polymerase binding site as occurs when RNA is the target. Thus, T7 RNA polymerase transcribes the newly replicated DNA into hundreds to thousands of RNA copies.[53]

Detection of *M. tuberculosis* in smear-positive respiratory samples, *Chlamydia trachomatis* in genital specimens, and HIV and cytomegalovirus (CMV) quantitation in blood are a few of the current applications for TAS.

Probe Amplification

In probe amplification procedures, the number of target nucleic acid sequences in a sample is not changed as it is in target amplification procedures like PCR. Rather, synthetic probes that are specific to the target sequences bind to the target where the probes themselves are amplified. There are three major procedures that are commercially available that involve the amplification of probe sequences: LCR (Abbott), strand displacement amplification (SDA) (Becton Dickinson), and Qβ replicase (Vysis).

Ligase Chain Reaction

LCR is a method for amplifying synthetic primers/probes complementary to target nucleic acid. LCR is similar to PCR, but there are a few differences. The entire target sequence must be known in order to prepare the oligonucleotide primers for LCR. In PCR, there can be a distance between the primers of up to hundreds of bases that is part of the amplified sequence. In LCR, by contrast, the primers bind adjacent to each other, separated by only one base. Instead of DNA polymerase synthesizing complementary DNA by extending the primers as occurs in PCR, DNA ligase is used in LCR to ligate the adjacent primers together. The ligated primers can then serve as a template for the annealing and ligation of additional primers. Because the product of LCR is ligated primer, LCR is better classified as a method of probe amplification rather than target amplification as the copy number of target molecules does not change.

LCR is similar to PCR in that it requires a thermal cycler to change the temperature to drive the different reactions. In LCR the tube is heated to denature the template. When the temperature is cooled, the primers anneal if the complementary sequence is present, and a thermostable ligase joins the two primers (Fig. 7-26). Even a 1–base pair mismatch at the ligation point will prevent ligation of the primers. Thus, LCR can be used to detect point mutations in a target sequence. The point mutation that occurs in the beta globulin of patients with sickle cell disease, as compared with normal beta globulin, was one of the first applications of LCR.[54]

Strand Displacement Amplification

SDA differs from most of the previously described amplification methods in that SDA is an **isothermal** amplification process, i.e., after an initial denaturation step, the reaction proceeds at one temperature.[55,56] SDA is more

Figure 7-26 Ligase chain reaction generates a signal by repeated ligation of probes complementary to specific sequences in the test DNA. One complementary oligomer is covalently attached to biotin for immobilization (square), and one has a signal-producing molecule (circle). The two oligomers will be ligated together only if the sequence of the target is complementary (left). The oligomers captured on a solid substrate by streptavidin will generate signal. If the sequence of the target is not complementary (right), the captured probe will not yield a signal.

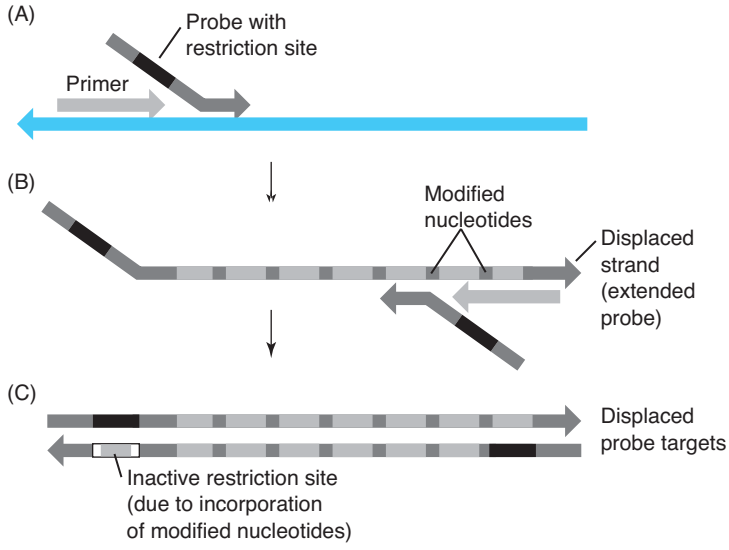

Figure 7-27 The first stage of SDA is the denaturation of the double-stranded target and annealing of primers and probes tailed with sequences including a restriction enzyme site (A; only one strand of the initial target is shown.) A second reaction (B) copies the probe, incorporating dATPaS and thereby inactivating the restriction site on the copied strand (C). This species is the target for amplification in the second stage of the reaction.

similar to LCR than PCR in that the major amplification products are the probes/primers and not the product of in vitro synthesis of target DNA. There are two stages to the SDA process. In the first stage (target generation), the target DNA is denatured by heating to 95°C. At each end of the target sequence, a primer and a probe bind close to each other (Fig. 7-27). The probes have a recognition sequence for a restriction enzyme. Exonuclease-deficient DNA polymerase derived from *E. coli* extends the primers, incorporating a modified nucleotide, 2′-deoxyadenosine 5′-O-(1-thiotriphosphate) (dATPaS). As the outer primers are extended, they displace the probes, which are also extended. A second set of complementary primers then bind to the displaced probes, and DNA polymerase extends the complementary primers, producing a double-stranded version of the probes. The probes are the target DNA for the next stage of the process.

The second stage of the reaction is the exponential probe/target amplification phase (Fig. 7-28). When the restriction enzyme is added to the double-stranded probe DNA, only one strand of the probe will be cut due to the dATPαS introduced in the extension reaction. This forms a nick in the DNA that is extended by DNA polymerase, simultaneously displacing the opposite strand. The displaced strand is also copied by primers that will restore the restriction site. As dATPαS is also used in the second-stage extension reactions, one strand of each new product will be resistant to the restriction enzyme, and the nicking/extension reaction can repeat without denaturation. Thus, the iterative process takes place at about 52°C without temperature cycling. The product of this amplification is millions of copies of the initial probe.

This method was first widely applied to detection of *M. tuberculosis*.[57] Methods using fluorescence polarization to detect the amplified target have been designed to test for *M. tuberculosis*[58] and *C. trachomatis*.[59] Addition of a fluorogenic probe to the reaction produces a fluorescent signal that corresponds to the amount of amplified target. This is the basis for the BDProbeTecET test for *M. tuberculosis*,[60] *C. trachomatis*, and *Neisseria gonorrhoeae*.[61]

Qβ Replicase

Qβ replicase is another method for amplifying probes that have specificity for a target sequence. The method is named for the major enzyme that is used to amplify probe sequences. Qβ replicase is a RNA-dependent RNA polymerase from the bacteriophage Qβ.[62] The target nucleic acid in this assay can be either DNA (which must first be denatured) or RNA.

The target nucleic acid is added to a well containing reporter probes. The reporter probes are RNA molecules that have specificity for the target sequence and also contain a promoter sequence (midivariant-1) that is recognized by the Qβ replicase. The reporter probes are allowed to hybridize to the template. The template with

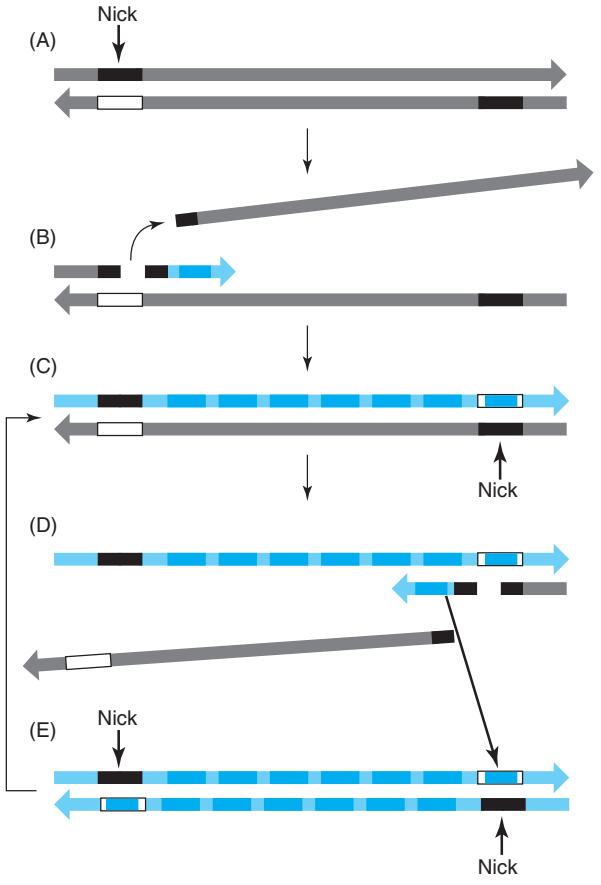

Figure 7-28 In the second phase of SDA, the target sequence is nicked by the restriction enzyme, generating a substrate for the polymerase, which extends the nick, displacing the opposite strand (B, D). The displaced strand is hybridized by a primer, producing another endonucleolytic target (C, E). The product of both reactions is a copy of the target with a hemisensitive restriction site (C, top). The reaction cycles of the strands are cut and extended.

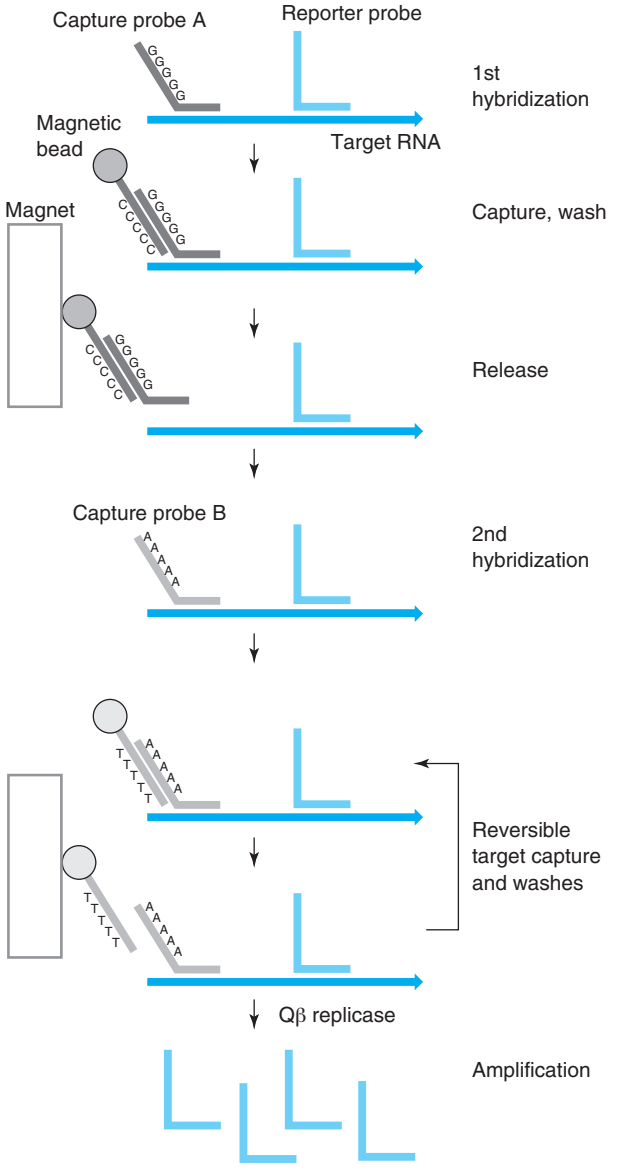

Figure 7-29 The Qβ replicase method proceeds through a series of binding and washing steps.[63,64] Probe bound to the purified template is then amplified by Qβ replicase. The resulting RNA can be detected by fluorometry using propidium iodide as a fluorescent label of the synthesized probe or by chromogenic methods.

bound probes is captured onto the side of the well using polyC capture probes and paramagnetic beads so that unbound reporter molecules can be washed away (Fig. 7-29). The template-probe complex is released from the polyC magnetic bead and bound to a different capture probe on a polyT paramagnetic bead. After a series of washes to remove unbound reporter probe, the template-probe complex is again released from the magnetic bead.

For the amplification step, the probe-bound template is mixed with the Qβ replicase, which replicates the probe molecules. This replication is very efficient with the generation of 10^6–10^9 RNA molecules/probe in less than 15 minutes. Because so many RNA molecules are produced, product detection can be achieved by colorimetric as well as real time fluorogenic methods. This assay can also be

quantitative by running a standard curve to determine the number of target molecules in the sample.

Qβ replicase has been used primarily to amplify the nucleic acid associated with infectious organisms, particularly mycobacteria,[63, 64] *Chlamydia,*[65] HIV,[66] and CMV,[67] but the assays are not commercially available in the United States at this time.

Signal Amplification

Signal amplification procedures differ from target amplification procedures in that the number of target sequences does not change; instead, large amounts of signal are bound to the target sequences that are present in the sample. Because the number of target sequences does not change, signal amplification procedures are inherently better at quantitating the amount of target sequences present in the clinical sample. Several signal amplification methods are available commercially.

Branched DNA Amplification

Chiron Corp. developed and markets the branched DNA (**bDNA**) amplification system. The target nucleic acid for this assay can be either DNA or RNA. A series of short oligomer probes are used to capture the target nucleic acid, and additional extender probes bind to the target nucleic acid and then to multiple reporter molecules,[68] loading the target nucleic acid with signal.

The bDNA signal amplification procedure is as follows. The target nucleic acid is released from the cells, the DNA is denatured if DNA is the target, and the target nucleic acid binds to capture probes that are fixed to a solid support (Fig. 7-30). Extender or preamplifier probes then bind to the captured target. The extender probes have sequences that are complementary to sequences in the target molecules as well as to sequences that are in the amplifier molecules. In the first-generation assay, the extender probes bind to a bDNA amplifier, which in turn bind multiple alkaline phosphatase-labeled nucleotides. Eight multimers or amplifiers, each with 15 branches, bind to each extender probe bound to the target. In the second- and third-generation assays, the extender probes bind preamplifiers, which in turn bind 14–15 amplifiers that can each bind to multiple alkaline phosphatase–labeled oligonucleotides (Fig. 7-31). Dioxetane is added as the substrate for the alkaline phosphatase, and chemi-

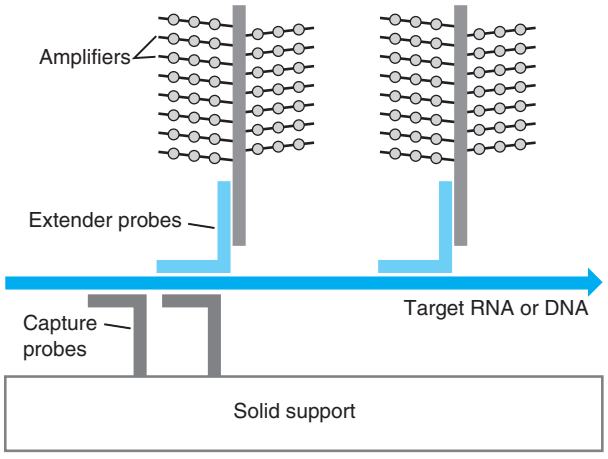

Figure 7-30 Branched DNA signal amplification of a single target. The target is captured or immobilized to a solid support by capture probes, after which extender probes and blocking probes create a stable cruciform structure with the amplifiers. Each amplifier has hybridization sites for 8–14 branches, which in turn bind substrate molecules for alkaline phosphatase.

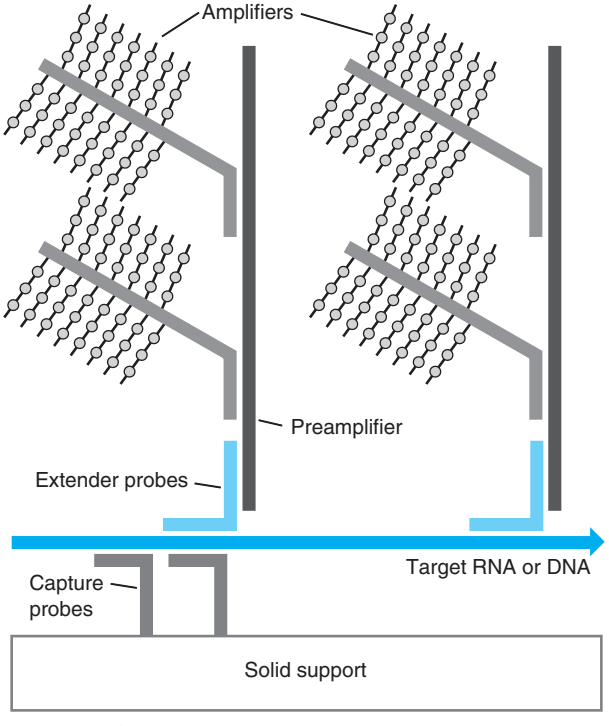

Figure 7-31 Second-generation bDNA assays use extender probes that bind multiple amplifiers, increasing the signal intensity and improving limits of detection.

luminescence is measured in a luminometer. This system has a detection limit of about 50 target mol/mL.[69]

There are several advantages to this method. First, there is less risk of carryover contamination resulting in a positive test in the bDNA assay than in PCR.[70] Second, multiple capture and extender probes can be incorporated that detect slightly different target sequences as occurs with different isolates of hepatitis C virus and HIV. By incorporating different probes that recognize slightly different sequences, multiple genotypes of the same virus can still be detected by the same basic system. Finally, requiring that multiple probes bind to the same target increases the specificity of the system. It is highly unlikely that all of the required probes would bind nonspecifically to an unrelated target and produce a signal. The bDNA signal amplification assay is currently available for the qualitative and quantitative detection of Hepatitis B Virus, Hepatitis C Virus, and HIV-1.[69–74]

Hybrid Capture Assays

Digene Diagnostics has marketed the **hybrid capture** assays primarily for the detection and molecular characterization of Human Papilloma Virus in genitourinary specimens.[75,76] It is also available for the detection of Hepatitis B Virus[77,78] and CMV.[79,80] In these assays, target DNA is released from cells and binds to single-stranded RNA probes (Fig. 7-32). The DNA/RNA hybrid has a unique structure that is recognized by antibodies. Antibodies bound to the surface of a microtiter well capture the DNA/RNA hybrids. Double-stranded DNA or single-stranded RNA will not bind to these antibodies. Captured hybrids are detected by the binding of alkaline phosphatase–conjugated anti–DNA/RNA hybrid antibodies in a typical sandwich assay. The substrate for the alkaline phosphatase is added, and chemiluminescence is measured. The sensitivity of this assay for Human Papilloma Virus has been reported to be about 1000 copies of viral DNA.[81] The hybrid capture assay is considered a signal amplification assay because the amount of target DNA is not amplified; rather, the DNA is isolated, bound to a "probe," and then label is bound to the target/probe hybrid molecule.

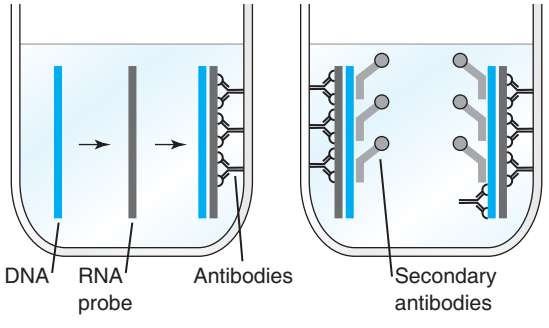

Figure 7-32 Hybrid capture starts with hybridization of the RNA probe to the denatured DNA target. The RNA/DNA hybrid is then bound by hybrid-specific immobilized antibodies. A secondary antibody bound to alkaline phosphatase generates signal in the presence of a chemiluminescent substrate (right).

Cleavage-Based Amplification

Cleavage-based amplification detects target nucleic acids by using a series of probes that bind to the target and overlap. Cleavase is an enzyme that has been isolated from bacteria that recognizes overlapping sequences of DNA and makes a cut ("cleaves") in the overlapping piece. In vivo, this activity is most likely important in repairing DNA. Third Wave Technologies has promoted this method as the basis of its Invader system. Targets for this form of amplification have been DNA polymorphisms, primarily for factor V Leiden mutation detection.[86, 87]

To start the amplification, the target nucleic acid is mixed with invader and signal probes (see Chapter 9, Fig. 9-26.). The invader probe and the signal probes bind at the target, with the 5′ end of the signal probe overlapping with the invader probe. Cleavase recognizes this overlap and cleaves the signal probe, which can act as an invader probe in the next step of the reaction. In the second step, a FRET probe is added that has sequences complementary to the cleaved signal probe. The 5′ end of the FRET probe has a reporter molecule that is located in proximity to a quencher molecule. As a result, the intact FRET probe does not produce a signal. The signal probe (now an invader probe) binds to the FRET probe, producing an overlapping region that is recognized by Cleavase. When Cleavase cuts the FRET probe in the overlapping region, it releases the reporter molecule from the quencher, resulting in the production of signal. The amount of sig-

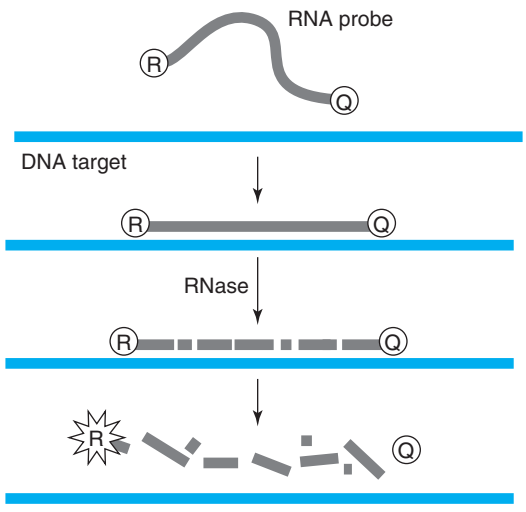

Figure 7-33 Cycling probe produces fluorescence only when the RNA probe binds to the DNA template. The RNA/DNA hybrid formed by the probe bound to the template is a substrate for RNase H, which digests the RNA probe and releases the reporter dye (R) from the vicinity of the quencher (Q).

nal can be quantitated and related directly to the amount of target molecules in the sample.

Cycling Probe

In the cycling probe method of amplification, target sequences are detected using a synthetic probe consisting of sequences of DNA-RNA-DNA. The probe binds to the target nucleic acid (Fig. 7-33). RNase H cleaves the RNA from the middle of the probe. This releases the DNA portions from the probes, freeing the template to bind to additional probe molecules. When the probe is digested, the reporter and quencher dye are separated, allowing fluorescence to escape from the reporter. The amount of fluorescence from the reporter dye (produced when the target is present) is measured as an indication of the presence of target molecules. Alternatively, the presence of chimeric probes that remain when target sequences are not present can also be measured. This method has been used to detect genes associated with antimicrobial resistance in bacteria such as methicillin resistance (*mecA*) in *Staphylococcus aureus* and vancomycin resistance (*vanA* and v*anB*) in *Enterococcus.*[88,89]

• STUDY QUESTIONS •

1. The final concentration of *Taq* polymerase is to be 0.01 units/μl in a 50 μl PCR. If the enzyme is supplied as 5 units/μl, how much enzyme would you add to the reaction?
   a. 1 μL
   b. 1 μL of a 1:10 dilution of *Taq*
   c. 5 μL of a 1:10 dilution of *Taq*
   d. 2 μL

2. Primer dimers result from:
   a. High primer concentrations
   b. Low primer concentrations
   c. High GC content in the primer sequences
   d. 3′ complementarity in the primer sequences

3. Which control is run to detect contamination?
   a. Negative control
   b. Positive control
   c. Molecular weight marker
   d. Reagent blank

4. Nonspecific extra PCR products can result from:
   a. Mispriming
   b. High annealing temperatures
   c. High agarose gel concentrations
   d. Omission of $MgCl_2$ from the PCR

5. Using which of the following is an appropriate way to avoid PCR contamination?
   a. High fidelity polymerase
   b. Hot-start PCR
   c. A separate area for PCR set up
   d. Fewer PCR cycles

6. How many copies of a target are made after 30 cycles of PCR?
   a. 2×30
   b. 2^{30}
   c. 30^2
   d. 30/2

7. What are the three steps of a standard PCR cycle?

8. Which of the following is a method for purifying a PCR product?
   a. Treat with uracil N glycosylase
   b. Add divalent cations
   c. Put the reaction mix through a spin column
   d. Add DEPC

9. In contrast to standard PCR, real-time PCR is:
   a. Quantitative
   b. Qualitative
   c. Labor-intensive
   d. Sensitive

10. In real-time PCR, fluorescence is *not* generated by which of the following?
    a. FRET probes
    b. TaqMan probes
    c. SYBR green
    d. *Tth* polymerase

11. Prepare a table that compares PCR, LCR, bDNA, TMA, Qβ Replicase, and Hybrid Capture with regards to the type of amplification, target nucleic acid, type of amplicon, and major enzyme(s) for each.

12. Examine the following sequence. You are devising a test to detect a mutation at the underlined position.

    5′ TATTTAGTTA TGGCCTATAC ACTATTTGTG AGCAAAGGTG ATCGTTTTCT GTTTGAGATT TTTATCTCTT GATTCTTCAA AAGCATTCTG AGAAGGTGAG ATAAGCCCTG AGTCTCAGCT ACCTAAGAAA AACCTGGATG TCACTGGCCA CTGAGGAGC TTTGTTTCAAC CAAGTCATGT GCATTTCCAC GTCAACAGAA TTGTTTATTG TGACAGTT**A**T ATCTGTTGTC CCTTTGACCT TGTTTCTTGA AGGTTTCCTC GTCCCTGGGC AATTCCGCAT TTAATTCATG GTATTCAGGA TTACATGCAT GTTTGGTTA AACCCATGAGA TTCATTCAGT TAAAAATCCA GATGGCGAAT 3′

    Design one set of primers (forward and reverse) to generate an amplicon containing the underlined base.

    The primers should be 20 bases long.

    The amplicon must be 100–150 bp in size.

    The primers must have similar melting temperatures (T_m), +/− 2°C.

    The primers should have no homology in the last three 3′ bases.

    a. Write the primer sequences 5′→ 3′ as you would if you were to order them from the DNA synthesis facility.
    b. Write the T_m for each primer that you have designed.

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Chapter 8

Lela Buckingham

Chromosomal Structure and Chromosomal Mutations

OUTLINE

OBJECTIVES

- Define mutations and polymorphisms.
- Distinguish the three types of DNA mutations: genome, chromosomal, and gene.
- Describe chromosomal compaction and the proteins involved in chromatin structure.
- Diagram a human chromosome, and label the centromere, q arm, p arm, and telomere.
- Illustrate the different types of structural mutations that occur in chromosomes.
- State the karyotype of a normal male and female.
- Identify the chromosomal abnormality in a given karyotype.
- Compare and contrast interphase and metaphase FISH analyses.
- Distinguish between the effects of balanced and unbalanced translocations on an individual and the individual's offspring.

The **human genome** is all of the genes found in a single individual. The human genome consists of 2.9 billion nucleotide base pairs of DNA organized into 23 chromosomes. As **diploid** organisms, humans inherit a **haploid** set of all their genes (23 chromosomes) from each parent, so that humans have two copies of every gene (except for some on the X and Y chromosomes). Each chromosome is a double helix of DNA, ranging from 246 million nucleotide base pairs in length in chromosome 1 (the largest) to 47 million nucleotide base pairs in chromosome 22 (the smallest; Table 8.1). Genetic information is carried on the chromosomes in the form of the order, or **sequence,** of nucleotides in the DNA helix. A **phenotype** is a trait or group of traits resulting from transcription and translation of these genes. The **genotype** is the DNA nucleotide sequence responsible for a phenotype.

Genotypic analysis is performed to confirm or predict phenotype. In the laboratory, some changes in chromosome structure and changes in chromosome number can be observed microscopically. Mutations at the nucleotide sequence level are detected using biochemical or molecular methods. Alterations of the DNA sequence may affect not only the phenotype of an individual but the progeny of that individual as well. The latter, **heritable changes,** are the basis for prediction of the phenotype in the next generation. The probability of inheritance of a phenotypic trait can be estimated using logical methods of mendelian genetics and statistics.

Table 8.1 **Sizes of Human Chromosomes in Base Pairs**

Chromosome	Millions of Base Pairs
1	246
2	244
3	199
4	192
5	181
6	171
7	158
8	146
9	136
10	135
11	134
12	132
13	113
14	100
15	90
16	82
17	76
18	64
19	64
20	47
21	47
22	49
X	154
Y	57

A transmissible (inheritable) change in the DNA sequence is a mutation or polymorphism. Although these terms are sometimes used interchangeably, they do have slightly different meanings based on population genetics. A DNA sequence change that is present in a relatively small proportion of a population is a **mutation**. The term, **variant**, may also be used, particularly to describe inherited sequence alterations, thus reserving the term mutation for somatic changes; for example, changes found only in tumor tissue. A change in the DNA sequence that is present in at least 1%–2% of a population is a **polymorphism**. Both mutations and polymorphisms may or may not produce phenotypic differences. Polymorphisms are casually considered mutations that do not severely affect phenotype; this is generally true, as any negative effect on survival and reproduction limits the persistence of a genotype in a population. Some polymorphisms are maintained in a population through a balance of positive and negative phenotype. The classic example is sickle cell anemia, a condition caused by a single-base substitution in the gene that codes for hemoglobin. The alteration is regarded as a mutation, but it is really a **balanced polymorphism**. In addition to causing abnormal red blood cells, the genetic alteration results in resistance to infection by *Plasmodium falciparum*; that is, resistance to malaria. The beneficial trait provides a survival and reproductive advantage that maintains the polymorphism in a relatively large proportion of the population. Examples of benign polymorphisms, that is, those with no selective advantage, are the ABO blood groups and the major histocompatibility complex (see Chapter 15). Polymorphisms used for human identification and paternity testing are discussed in Chapter 10.

DNA mutations can affect a single nucleotide or millions of nucleotides, even whole chromosomes, and thus can be classified into three categories. **Gene mutations** affect single genes and are often, but not always, small changes in the DNA sequence. **Chromosome mutations** affect the structures of entire chromosomes. These

changes require movement of large chromosomal regions either within the same chromosome or to another chromosome. **Genome mutations** are changes in the number of chromosomes. A cell or cell population with a normal complement of chromosomes is **euploid**. Genome mutations result in cells that are **aneuploid**. Aneuploidy is usually (but not always) observed as increased numbers of chromosomes, because the loss of whole chromosomes is not compatible with survival. A single copy of each chromosome (23 in humans) is a **haploid** complement. Humans are normally **diploid**, with two copies of each chromosome. Aneuploidy can result when there are more than two copies of a single chromosome or when there are multiple copies of one or more chromosomes. Down's syndrome is an example of a disease resulting from aneuploidy, where there are three copies, or **triploidy**, of chromosome 21.

Detection of mutations in the laboratory ranges from direct visualization of genome and chromosomal mutations under the microscope to indirect molecular methods to detect single-base changes. Methods used for detection of genome and chromosomal mutations are discussed in this chapter. Methods to detect gene mutations are described in Chapter 9.

Chromosomal Structure And Analysis

Chromosomal Compaction and Histones

An important concept in the understanding of chromosomes is that chromosome behavior is dependent on chromosome structure as well as DNA sequence.[1] Genes with identical DNA sequences will behave differently, depending on their chromosomal location or the surrounding nucleotide sequence. For example, certain functional features, such as the **centromere** (where the chromosome attaches to the spindle apparatus for proper segregation during cell division), are not defined by specific DNA sequences.[2] It is a well-known phenomenon that a gene inserted or moved into a different chromosomal location may be expressed (transcribed and translated) differently than it was in its original position. This is called **position effect**.

A eukaryotic chromosome is a double helix of DNA. A cell nucleus contains 4 cm of double helix. This DNA must be compacted, both to fit into the cell nucleus and for accurate segregation in mitosis. There is an 8000-fold compaction of an extended DNA double helix to make a metaphase chromosome (Fig. 8-1).[3] Winding of DNA onto **histones** is the first step. Histones are the most abundant proteins in cells. There are five histones: H1, H2a, H2b, H3, and H4. Approximately 160–180 bp of DNA is wrapped around a set of 8 histone proteins (2 each of H2a, H2b, H3 and H4) to form a **nucleosome**. Nucleosomes can be seen by electron microscopy as 100-Å beadlike structures that are separated by short strands of free double helix (Fig. 8-2). DNA wrapped around histones forms a "bead-on-a-string" arrangement that comprises the 10-nm or **10-micron fiber**. The 10-micron fiber is further coiled around histone H1 into a thicker and shorter 30-nm or **30-micron fiber**. The 30-nm interphase fibers represent the "resting state" of DNA. The fibers are locally relaxed into 10-nm fibers for DNA metabolism as required during the cell cycle. These fibers are looped onto protein scaffolds to form 300-nm fibers; before entry into the M phase of the cell cycle (mitosis), the looped fibers are wound into 700-nm solenoid coils.[4] The 700-nm coils are compacted into the 1400-nm fibers that can be seen in metaphase nuclei and in karyotypes.

Advanced Concepts

The structure of metaphase chromosomes is maintained by more than just histones. Metaphase chromatin is one-third DNA, one-third histones, and one-third nonhistone proteins. Nonhistone protein complexes, termed condensin I and condensin II, are apparently required for maintenance of mitotic chromosome structure.[28]

Historical Highlights

Before 1943, histones were thought to contain genetic information. Their function was later thought to be structural. It is now known that modification of histones, through acetylation, methylation, phosphorylation, or ubiquitination, plays a role in other cellular functions such as recombination, replication, and gene expression.[32]

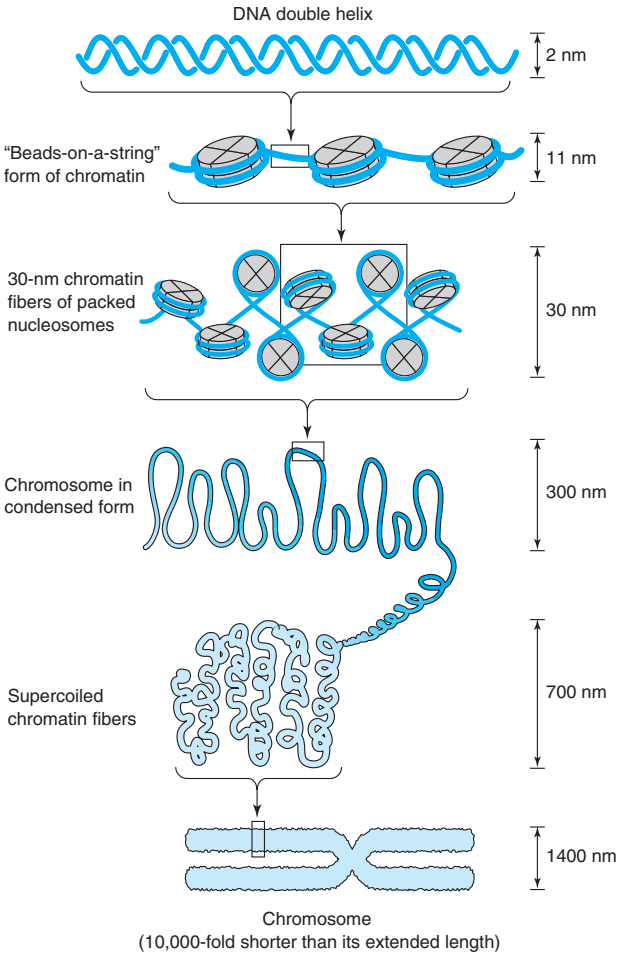


Figure 8-1 DNA compaction into metaphase chromosomes. (From B Alberts, Molecular Biology of the Cell, 4th edition, Garland Science, New York, 2002.)

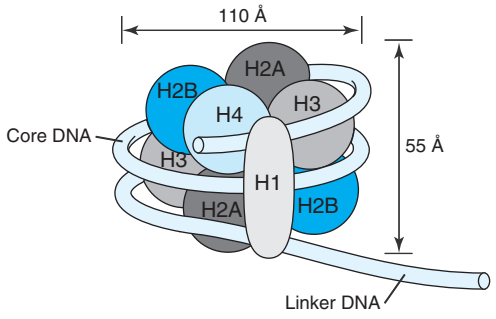


Figure 8-2 DNA wrapped around eight histone proteins (2 each of histone 2A, 2B, 3, and 4) forms a nucleosome. A further association with histone H1 coils the nucleosomal DNA into a 30-nm fiber.

When the DNA is relaxed into 10-micron fibers for transcription or replication, the placement of nucleosomes along the double helix can be detected using nucleases (e.g., Mung bean nuclease, or DNase I). These enzymes cut the double helix in the **linker region**, the part of the double helix that is exposed between the histones.

To make 30-nm chromatin fibers, the internucleosomal DNA is associated with histone H1, and the beaded structure is wound into a solenoid coil. Loss of this level of organization is the first classic indicator of **apoptosis**, or programmed cell death. The 30-nm fibers are uncoiled, and the exposed linker DNA between the nucleosomes becomes susceptible to digestion by intracellular nucleases. The DNA wrapped into the nucleosomes remains intact so that DNA isolated from apoptotic cells contains "ladders," or multiples of discreet multiples of ~180 bp. These ladders can be resolved by simple agarose electrophoresis (Fig. 8-3). The remainder of the proteins involved in DNA compaction are the **nonhistone proteins**.

Chromosome topology (state of compaction of the DNA double helix) affects gene activity; for instance in chromosome X inactivation in females. More highly compacted DNA is less available for RNA transcription. Maintenance of the more highly compacted state of DNA in closed chromatin, or **heterochromatin** (in contrast to open chromatin, or **euchromatin**), throughout interphase may require special proteins called condensin proteins or condensin-like protein complexes.

Advanced Concepts

Members of a family of proteins called SMC proteins control chromosome condensation in eukaryotes and other aspects of chromosome behavior, including chromosome segregation in prokaryotes. Two of the SMC proteins, XCAP-C and XCAP-E, first isolated from frog eggs,[29] are integral parts of the **condensin complex**, a protein scaffold structure that can be isolated from both mitotic and interphase cells. This complex in the presence of topoisomerase can wrap DNA around itself in an ATP-driven reaction. Although the exact role of this complex in condensation and decondensation is not yet completely defined, this ability to change chromosome architecture is a significant feature of DNA metabolism.

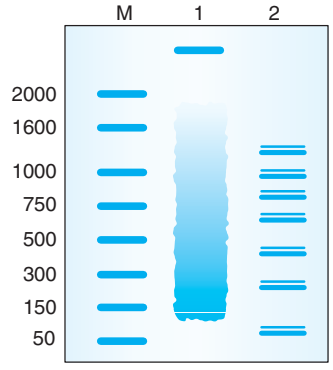


Figure 8-3 Apoptotic DNA (lane 2) is characterized by the ladder seen on gel electrophoresis. This is in contrast to degraded DNA from necrotic cells (lane 1). Lane M contains molecular weight markers.

Chromosome Morphology

Mitotic chromosomes have been distinguished historically by their relative size and centromere placement. As previously stated, the **centromere** is the site of attachment of the chromosome to the spindle apparatus. The connection is made between microtubules of the spindle and a protein complex, called the **kinetochore**, that assembles at the centromere sequences (Fig. 8-4). At the nucleotide level, the centromere is composed of a set of highly repetitive **alpha satellite** sequences.[5] Microscopically, the centromere appears as a constriction in each compacted metaphase chromosome. Chromosomes are **metacentric**, **submetacentric, acrocentric**, or **telocentric**, depending on the placement of the centromere (Fig. 8-5). The placement of the centromere divides the chromosome into arms.

There are no telocentric human chromosomes. Human chromosomes are acrocentric or submetacentric and so have long and short arms (Table 8.2). The long arm of a chromosome is designated **q**, and the short arm is designated **p**. Acrocentric chromosomes have a long arm length:short arm length ratio of from 3:1 to 10:1. Chromosomes 13 to 15, 21, and 22 are acrocentric.

Advanced Concepts

Some plants and insects have **holocentric** chromosomes. During cell division, these chromosomes form kinetochores along their entire length.

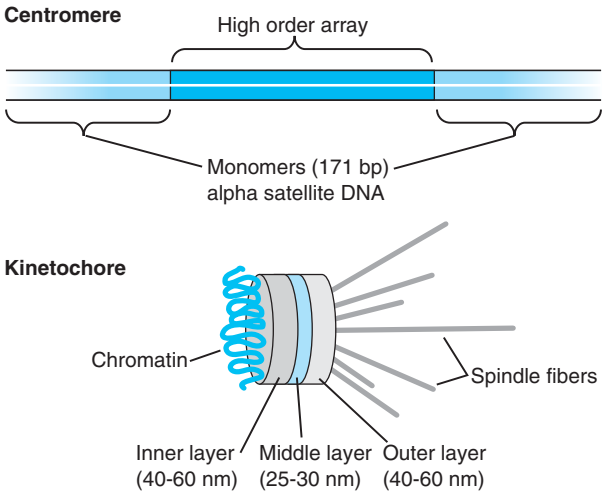


Figure 8-4 The centromere (top) consists of tandem repeats of 171 base pair sequences flanking sets of single repeat units, or monomers repeated in groups in a higher order array. The kinetochore (bottom) is a protein structure that connects the centromeres to the spindle apparatus.

Visualizing Chromosomes

Conventional cytological stains, such as Feulgen's, Wright's, and hematoxylin, have been used to visualize chromosomes. An advance in the recognition of individual chromosomes was the demonstration that fluorescent stains and chemical dyes can react with specific chromosome regions. This region-specific staining results in the formation of band patterns where portions of the chro-

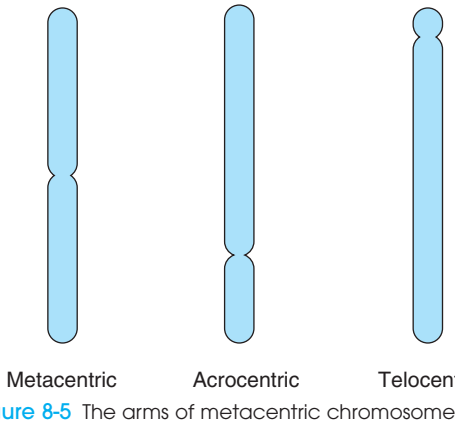


Figure 8-5 The arms of metacentric chromosomes (left) are of equal size. Acrocentric chromosomes (center) divide the chromosome into long arms and short arms. Telocentric centromeres (right) are at the ends of the chromosome.

Table 8.2 Classification of Chromosomes by Size and Centromere Position

Group	Chromosomes	Description
A	1, 2	Large metacentric
	3	Large submetacentric
B	4, 5	Large submetacentric
C	6–12, X	Medium-sized submetacentric
D	13–15	Medium-sized acrocentric with satellites
E	16	Short metacentric
	17, 18	Short submetacentric
F	19, 20	Short metacentric
G	21, 22	Short acrocentric with satellites
	Y	Short acrocentric

mosome accept or reject the stain. For cytogenetic analysis, this allows unequivocal identification of every chromosome and the direct detection of some chromosomal abnormalities. Underlying the region-specific staining is the implication that the reproducible staining patterns occur as a result of defined regional ultrastructures of the mitotic chromosomes.

When chromosomes are stained with the fluorescent dyes quinacrine and quinacrine mustard, the resulting fluorescence pattern visualized after staining is called **Q banding** (Fig. 8-6). This method was first demonstrated in 1970 by Caspersson, Zech, and Johansson.[6] The results of this work confirmed that each human chromosome could be identified by its characteristic banding pattern. Q banding gives a particularly intense staining of the human Y chromosome and thus may also be used to distinguish the Y chromosome in interphase nuclei. Because Q banding requires a fluorescent microscope, it is not as widely used as other stains that are detectable by light microscopy.

The chemical dye Giemsa stains in patterns, or **G bands**, similar to those seen in Q banding. The appearance of G banding differs, depending on the treatment of the chromosomes before staining.[7] Mild treatment (2× standard saline citrate for 60 minutes at 60°C) yields the region-specific banding pattern comparable to that seen with fluorescent dyes. Use of trypsin or other proteolytic agents to extract or denature proteins before Giemsa staining was found to map structural aberrations more clearly and is the most commonly used staining method

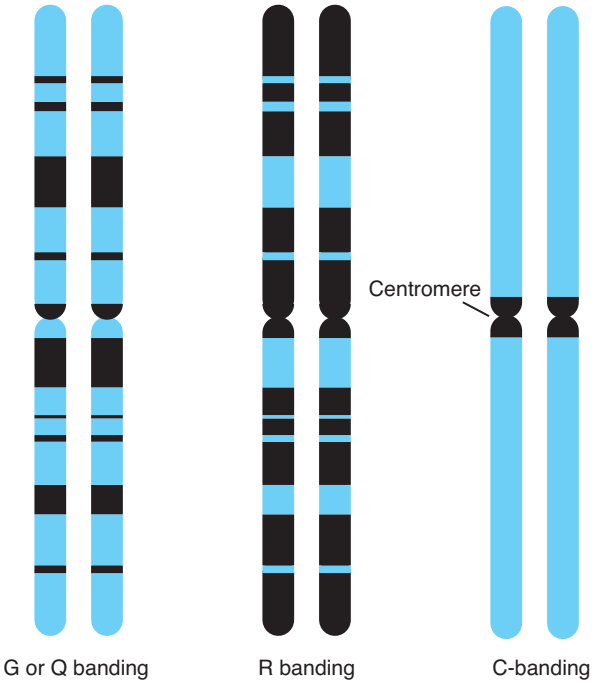


Figure 8-6 Reproducible staining patterns on chromosomes are used for identification and site location. Heterochromatin stains darkly by G or Q banding (left); euchromatin stains darkly by R banding (center); C banding stains centromeres (right).

for analyzing chromosomes.[8,9] G bands can also be produced by Feulgen staining after treatment with DNase I.[10] The number of visualized bands can be increased from about 300 to about 500 per chromosome by staining chromosomes before they reach maximal metaphase condensation. This is called **high-resolution banding**.

Harsher treatment of chromosomes (87°C for 10 minutes, then cooling to 70°C) before Giemsa staining will produce a pattern opposite to the G banding pattern called **R banding**.[11] R bands can also be visualized after staining with acridine orange.[12]

Alkali treatment of chromosomes results in centromere staining, or **C banding.**[13] Centromere staining is absent in G band patterns. C bands may be associated with heterochromatin, the "quiet," or poorly, transcribed sequences along the chromosome that are also present around centromeres. In contrast, euchromatin, which is relatively rich in gene activity, may not be stained as much as heterochromatin in C banding.

Advanced Concepts

The correlation between heterochromatin and staining may also hold for noncentromeric G and Q bands. This association is complicated, however, because a variety of procedures and stains produce identical banding patterns. The correlation of staining with heterochromatin is contradicted by observations of the X chromosome. Although one X chromosome is inactive and replicates later than the active X in females, both X chromosomes stain with equal pattern and intensity. Staining differences, therefore, must be due to other factors. Possible explanations for differential interactions with dye include differences in DNA compaction, sequences, and DNA-associated nonhistone proteins.

Nucleolar organizing region staining (**NOR staining**) is another region-specific staining approach. Chromosomes treated with silver nitrate will stain specifically at the constricted regions, or stalks, on the acrocentric chromosomes.

Staining of chromosomes with 4′,6-diamidino-2-phenylindole (**DAPI**) was first described in 1976 as a way to detect mycoplasmal contamination in cell cultures.[14] DAPI binds to the surface grooves of double-stranded DNA and fluoresces blue under ultraviolet light (353-nm wavelength). DAPI can be used to visualize chromosomes as well as whole nuclei.

Chromosome banding facilitates detection of small deletions, insertions, inversions, and other abnormalities and the identification of distinct chromosomal locations.

Advanced Concepts

Chromosomes can be prestained with the DNA-binding oligopeptide distamycin A to enhance chromosomal distinctions.[30,31] DAPI/distamycin A staining is useful in identifying pericentromeric breakpoints in chromosomal rearrangements and other rearrangements or chromosomes that are too small for standard banding techniques.

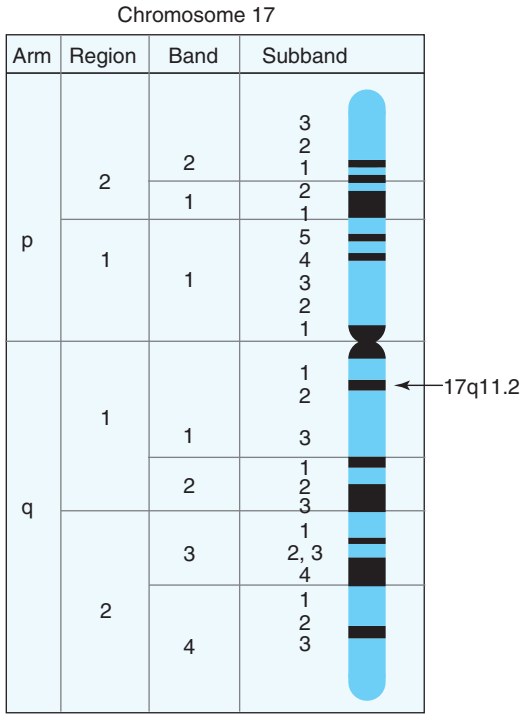


Figure 8-7 Identification of chromosomal location by G-band patterns. Locations are designated by the chromosome number 17 in this example, the arm q, the region 1, the band 1 and the sub-band 2.

For this purpose, the reproducible G-banding pattern has been ordered into regions, comprising bands and sub-bands. For example, in Figure 8-7 a site on the long arm (q) of chromosome 17 is located in region 1, band 1, sub-band 2, or 17q11.2.

Detection Of Genome And Chromosomal Mutations

Karyotyping

Genome mutations, or aneuploidy, can be detected by indirect methods, such as flow cytometry and more directly by karyotyping. A **karyotype** is the complete set of chromosomes in a cell. Karyotyping is the direct observation of metaphase chromosome structure by arranging metaphase chromosomes according to size. Karyotyping requires collecting living cells and growing them in culture in the laboratory for 48–72 hours. Cell division is stimu-

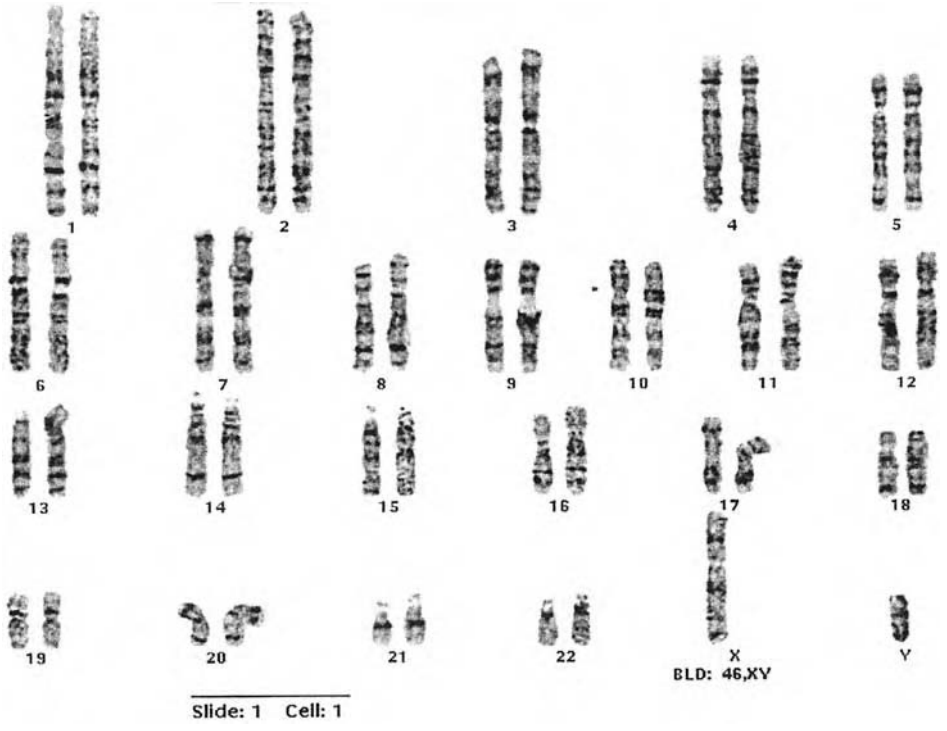


Figure 8-8 A normal male karyotype. There are 22 sets of autosomes, one inherited from each parent, and one pair of sex chromosomes, XY. This karyotype is designated 46, XY.

lated by addition of a **mitogen**, usually phytohemagglutinin. Dividing cells are then arrested in metaphase with colcemid, an inhibitor of microtubule (mitotic spindle) formation. The chromosomes in dividing cells that arrest in metaphase will yield a **chromosome spread** when the cell nuclei are disrupted with hypotonic saline. The 23 pairs of chromosomes can then be assembled into an organized display, or **karyotype**, according to their size and centromere placement (Fig. 8-8). Aneuploidy can be observed affecting several chromosomes[15] (Fig. 8-9) or a single chromosome (Fig. 8-10).

Karyotyping can also detect chromosomal mutations such as **translocations**, which are the exchange of genetic material between chromosomes. Translocations can be of several types. In **reciprocal** translocations, parts of two chromosomes exchange, i.e., each chromosome breaks, and the broken chromosomes reassociate or recombine with one another. When this type of translocation does not result in gain or loss of chromosomal material, it is **balanced** (Fig. 8-11, Fig. 8-12). Balanced translocations can

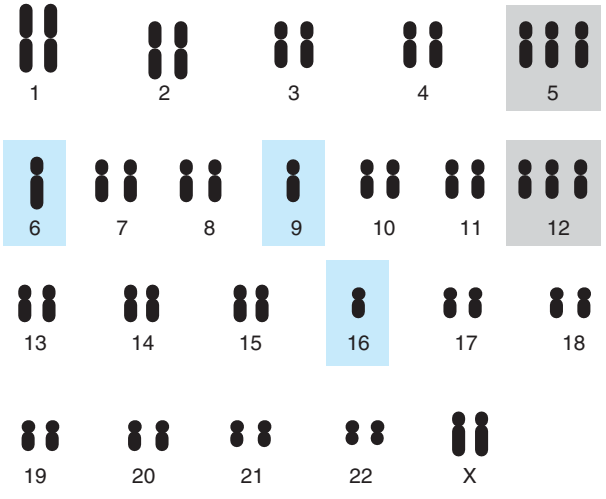


Figure 8-9 Aneuploidy involving multiple chromosomes. Chromosomes 5 and 12 are triploid; chromosomes 6, 9, and 16 are monoploid.

■ Figure 8-10 Aneuploidy involving the Y chromosome (XYY syndrome). This is designated 47,XYY.

occur, therefore, without phenotypic effects. Balanced translocations in germ cells (cells that give rise to eggs or sperm) can, however, become **unbalanced** by not assorting properly during meiosis; as a result, they affect the phenotype of offspring. A **robertsonian** translocation involves the movement of most of one entire chromosome to the centromere of another chromosome (Fig. 8-13). This type of translocation can also become unbalanced during reproduction, resulting in a net gain or loss of chromosomal material in the offspring.

Other types of chromosome mutations that are sometimes visible by karyotyping are shown in Figure 8-14. A **deletion** is a loss of chromosomal material. Large deletions covering millions of base pairs can be detected using karyotyping; smaller **microdeletions** are not always easily seen using this technique. An **insertion** is a gain of chromosomal material. The inserted sequences can arise from duplication of particular regions within the affected chromosome or from fragments of other chromosomes. As with deletions, altered banding patterns and a change in the size of the chromosomes can indicate the occurrence of this event. **Inversions** result from excision, flipping, and reconnecting chromosomal material within

Historical Highlights

The first chromosome mutations were visualized in the 1960s in leukemia cells. Peter Nowell and colleague David Hungerford observed an abnormally small chromosome 22 in leukemia cells, which they labeled the "Philadelphia" chromosome. A few years later, Janet Rowley, using chromosome banding, noted that tumor cells not only lost genetic material, they exchanged it. In 1972 she first described the translocation between chromosomes 8 and 21, t(8;21) in patients with acute myeloblastic leukemia. In that same year, she demonstrated that the Philadelphia chromosome was the result of a reciprocal exchange between chromosome 9 and chromosome 22. She went on to identify additional reciprocal translocations in other diseases, the t(14;18) translocation in follicular lymphoma and the t(15;17) translocation in acute promyelocytic leukemia. This was the first evidence that cancer had a genetic basis.

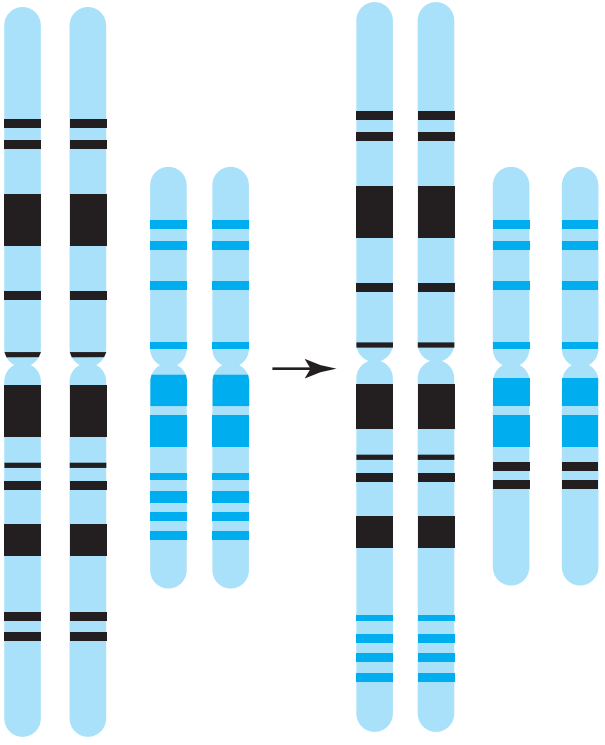


Figure 8-11 A balanced reciprocal translocation.

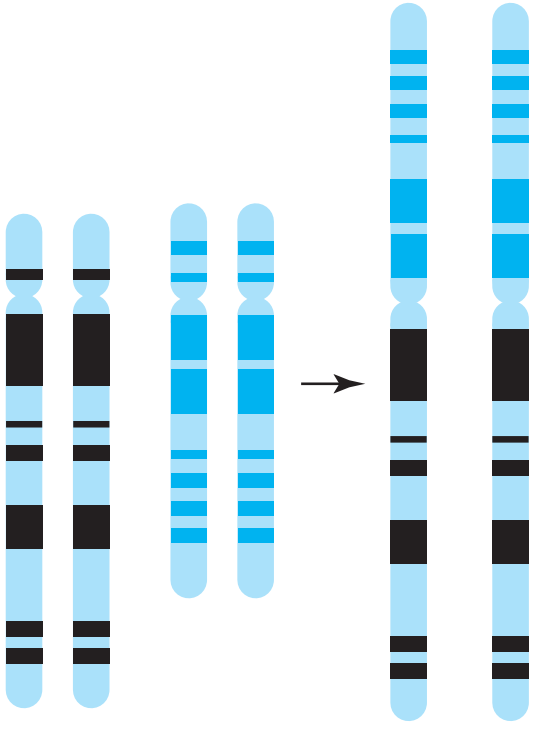


Figure 8-13 A robertsonian translocation.

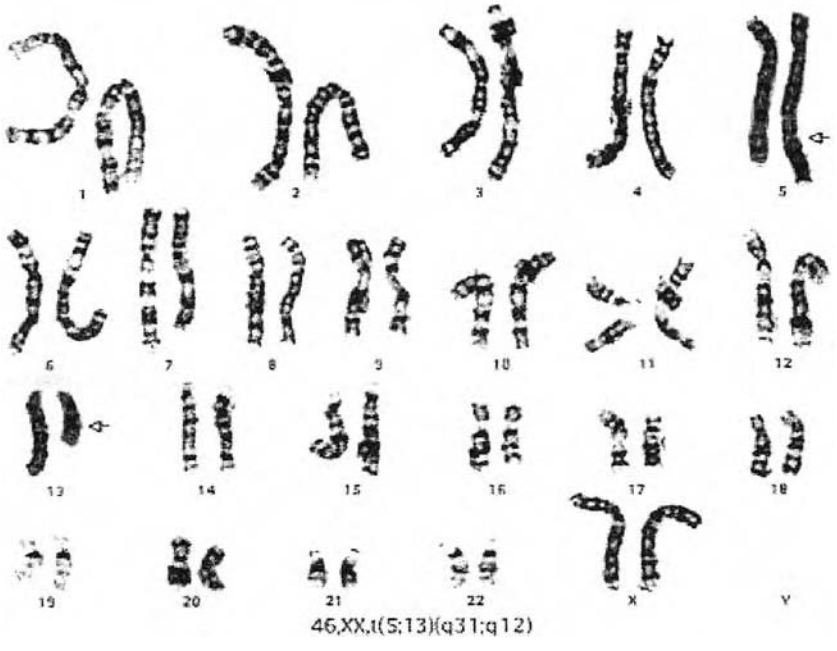


Figure 8-12 A karyotype showing a balanced reciprocal translocation between chromosomes 5 and 13. This is designated 46, XX,t(5;13).

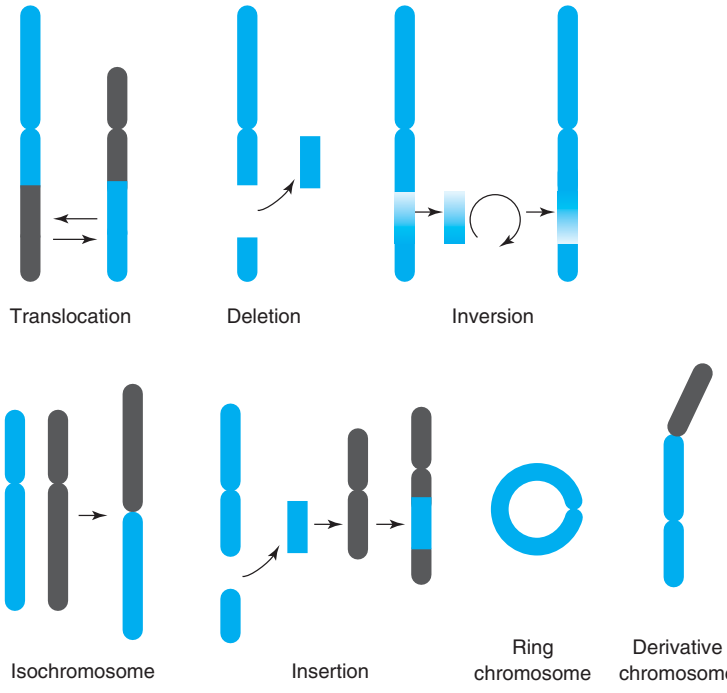


Figure 8-14 Chromosome mutations involving alterations in chromosome structure.

the same chromosome. **Pericentric inversions** include the centromere in the inverted region, whereas **paracentric inversions** involve sequences within one arm of the chromosome. An **isochromosome** is a metacentric chromosome that results from transverse splitting of the centromere during cell division. Transverse splitting causes two long arms or two short arms to separate into daughter cells instead of normal chromosomes with one long arm and one short arm. The arms of an isochromosome are, therefore, equal in length and genetically identical. A **ring chromosome** results from deletion of genetic regions from both ends of the chromosome and a joining of the ends to form a ring. A **derivative chromosome** is an abnormal chromosome consisting of translocated or otherwise rearranged parts from two or more unidentified chromosomes joined to a normal chromosome.

Results of karyotyping analyses are expressed as the number of chromosomes/nucleus (normal is 46), the sex chromosomes (normal is XX or XY), followed by any genetic abnormalities observed. A normal karyotype is 46, XX in a female or 46, XY in a male. 46,XX,del(7)(q13) denotes a deletion in the long arm q of chromosome 7 at region 13. 46,XY,t(5;17)(p13.3;p13) denotes a translocation between the short arms of chromosomes 5 and 17 and region 13, band 3, and region 13, respectively. 47,XX+21 is the karyotype of a female with Down's syndrome resulting from an extra chromosome 21. Klinefelter's syndrome is caused by an extra X chromosome in males; for example, 47,XXY. Table 8.3 shows a list of some of the terms used in expressing karyotypes.

Table 8.3 **A List of Descriptive Abbreviations**

Abbreviation	Indication
+	gain
−	loss
del	deletion
der	derivative chromosome
dup	duplication
ins	insertion
inv	inversion
I, iso	isochromosome
mat	maternal origin
pat	paternal origin
r	ring chromosome
t	translocation
tel	telomere (end of chromosome arm)

Fluorescence In Situ Hybridization

Interphase FISH

Fluorescence in situ hybridization (FISH) is a widely used method to detect protein, RNA as well as DNA structures in place in the cell or in situ. For cytogenetic analysis, fixed cells are exposed to a probe. The probe is a 60-200–kb fragment of DNA attached covalently to a fluorescent molecule. The probe will hybridize or bind to its complementary sequences in the cellular DNA. In interphase FISH, the bound probe can be visualized under a fluorescent microscope in the nucleus of the cell. Probes are designed to be specific to a particular chromosome or chromosomal regions so that the image under the microscope will correlate with the state of that chromosome or region. For example, a probe to any unique region on chromosome 22 should yield an image of two signals per nucleus, reflecting the two copies of chromosome 22 in the somatic cell nucleus (Fig. 8-15). A deletion or duplication of the region that is hybridized to the probe will result in a nucleus with only one signal or more than two signals, respectively. Multiple probes spanning large regions are used to detect regional deletions.[16,17] One advantage of interphase FISH is that growth of cells in culture is not required. FISH methods are, therefore, used commonly to study prenatal samples, tumors, and hematological malignancies, not all of which are conveniently brought into metaphase in culture.

Translocations or other rearrangements can be detected using probes of different colors complementary to regions on each chromosome taking part in the translocation (Fig. 8-16). A translocated chromosome will combine the two probe colors with a loss of one of each signal. Analysis of translocation signals is sometimes complicated by false signals that result from two chromosomes landing close to one another in the nucleus, such that the bound probes give a signal similar to that exhibited by a translocation. These false signals can often be distinguished from true translocations by the size of the fluorescent image, but this distinction requires a trained eye. Accounting for false-positive signals as background noise limits the sensitivity of this assay.

The sensitivity of interphase FISH analysis can be increased using dual color probes, or **dual fusion probes**. These probes, 0.8–1.5 Mb in size, are designed to bind to regions spanning the breakpoint of both translocation

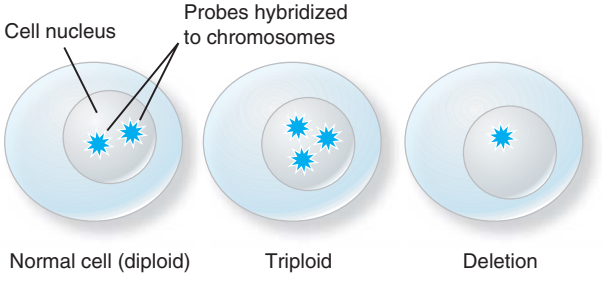


Figure 8-15 FISH analysis for a normal diploid cell (left), triploidy (center), and deletion (right).

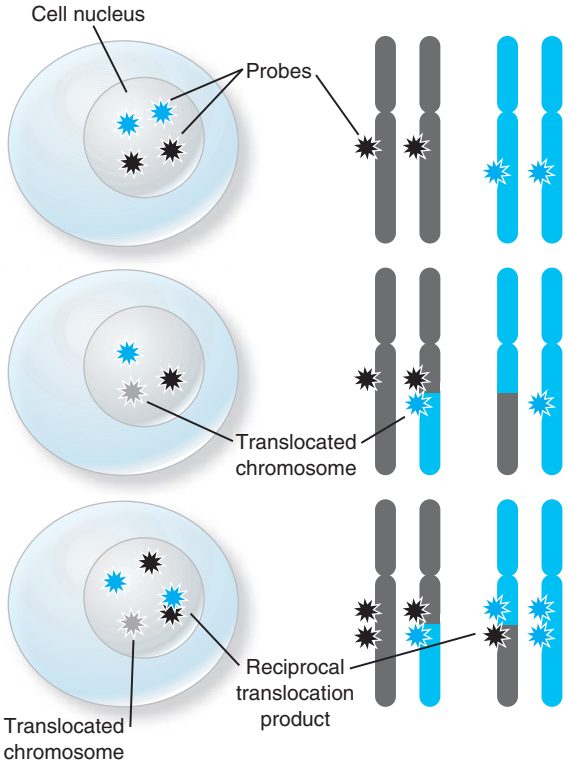


Figure 8-16 FISH analysis using distinct probes to detect a translocation. A normal nucleus has two signals from each probe (top). A translocation involving the two chromosomes combines the two probe colors (middle). Dual-fusion probes confirm the presence of the translocation by also giving a signal from the reciprocal breakpoint (bottom).

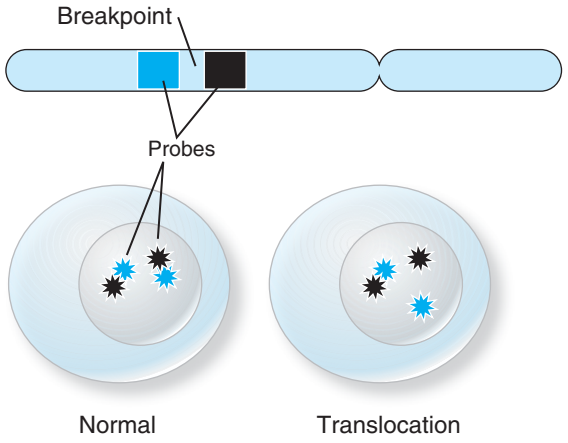


Figure 8-17 Break-apart probes bind to the chromosome flanking the translocation breakpoint region. Normal cells will display the combination signal (bottom left), and a translocation will separate the probe signals (bottom right).

partners. A translocation will be observed as a signal from both the translocation junction and the reciprocal of the translocation junction; e.g., t(9;22) and t(22;9); see Fig. 8-16. Dual color **break-apart probes,** 0.6–1.5 Mb**,** are another approach to lower background as well as to identify translocation events where one chromosome can recombine with multiple potential partners. These probes are designed to bind to the intact chromosome flanking the translocation breakpoint. When a translocation occurs, the two probes separate (Fig. 8-17). Sometimes called tri-FISH, break-apart probes are not the same as tricolor probes (see below).

Centromeric probes (CEPs) are designed to hybridize to highly repetitive alpha satellite sequences surrounding centromeres. These probes detect aneuploidy of any chromosome. Combinations of centromeric probes and region-specific probes are often used to confirm deletions or amplifications in specific chromosomes. Addition of a CEP to dual color probes serves as a control for amplification or loss of one of the chromosomes involved in the translocation. This combination of CEP and dual color probes comprises a **tricolor probe**. For example, the IGH/MYC CEP 8 Tri-color Dual Fusion Translocation Probe (Vysis) is a mixture of a 1.5-Mb–labeled probe, complementary to the immunoglobulin heavy chain region (IGH) of chromosome 14, an approximately 750-kb distinctly labeled probe complementary to the *myc* gene on chromosome 8 and a CEP to chromosome 8. The IGH probe contains sequences homologous to the entire IGH locus as well as sequences extending about 300 kb beyond the 3′ end of the IGH locus. The *myc* probe extends approximately 400 kb upstream and about 350 kb 3′ beyond the *myc* gene. CEP 8 targets chromosome 8 alpha satellite sequences and serves as a control to detect amplification of *myc* or loss of the chromosome 8 derivative resulting from the translocation.

Each chromosome arm has a unique set of repeat sequences located just before the end of the chromosome, called the **telomere** (Fig. 8-18). These sequences have been studied for the development of a set of DNA probes specific to the telomeres of all human chromosomes. **Telomeric probes** are useful for the detection of chromosome structural abnormalities such as cryptic translocations or small deletions that are not easily visualized by standard karyotyping.

Because interphase cells for FISH do not require culturing of the cells and stimulating division to get metaphase spreads, as is required for standard karyotyping, interphase FISH is faster than methods using metaphase cells and is valuable for analysis of cells that do not divide well in culture, including fixed cells.[18,19] Furthermore, as 200–500 cells can be analyzed microscopically using FISH, the sensitivity of detection is higher than that of metaphase procedures, which commonly examine 20 spreads. A limitation of FISH, however, is the inability to identify chromosomal changes other than those at the specific binding region of the probe. In contrast, karyotyping is a more generic method that can detect any chromosomal change that causes

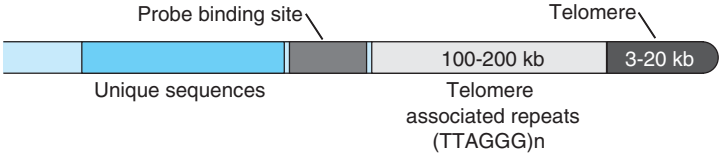


Figure 8-18 The binding sites for telomeric probes are unique sequences just next to the telomeric associated repeats and telomeric repeat sequences at the ends of chromosomes.

changes in chromosomal size, number, or banding pattern within the sensitivity limits of the procedure.

Preparation of the sample is critical in interphase FISH analysis, both to permeabilize the cells for optimal probe-target interaction and to maintain cell morphology.[20] Optimal results are obtained if fresh interphase cells are incubated overnight (aging) after deposition on slides. After aging overnight, cells are treated with protease to minimize interference from cytoplasmic proteins and fixed with 1% formaldehyde to stabilize the nuclear morphology. Before DNA denaturation, the cells are dehydrated in graded concentrations of ethanol. Paraffin-embedded tissues must be dewaxed in xylene before protease and formaldehyde treatment.

The quality of the probe should also be checked and its performance validated before use. Fluorescent probes (DNA with covalently attached fluorescent dyes) are usually purchased from vendors, which may also supply compatible hybridization reagents and controls. Nevertheless, it is recommended that the probe performance be observed on control tissue before use on patient samples. Under a fluorescent microscope with the appropriate color distinction filters, the signal from the probe should be bright, specific to the target in the cell nuclei, and free of high background. Probes differ in their signal characteristics and intensities; the technologist should become familiar with what to expect from a given probe on different types of tissues.

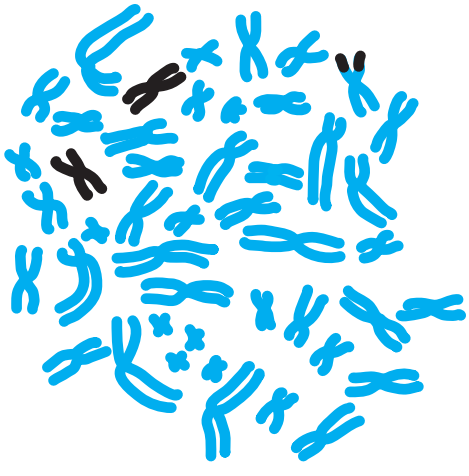
Similar to Southern and Northern blotting procedures, both probe and target must be denatured prior to hybridization. The amount of time taken to hybridize and use Cot-1 DNA (to reduce nonspecific binding) or facilitators such as dextran sulfate (to increase the effective probe concentration) depend on the sequence complexity of the probe (see Chapter 6). A probe 10 ng–1 μg may be used in a hybridization volume of 3-10 μL. The hybridization of the probe on the target cells should be performed at 37-42°C in a humidified chamber. The slides are cover-slipped and sealed to optimize the hybridization conditions.

Following hybridization and the removal of unbound probe by rinsing in Coplin jars, the sample is observed microscopically. The probe signals should be visible from entire intact nuclei. Although adequate numbers of cells must be visible, crowded cells where the nuclei and signals overlap do not yield accurate results. Furthermore, different tissue types have different image qualities and characteristics that must also be taken into account when assessing the FISH image.

Metaphase FISH

Metaphase analysis has been enhanced by the development of fluorescent probes that bind to metaphase chromosomal regions or to whole chromosomes. Probes that cover the entire chromosome, or **whole chromosome paints**, are valuable for detecting small rearrangements that are not apparent by regular chromosome banding (Fig. 8-19). By mixing combinations of five fluors and using special imaging software, **spectral karyotyping** can distinguish all 23 chromosomes by chromosome-specific colors.[21] This type of analysis can be used to detect abnormalities that affect multiple chromosomes as is sometimes found in cancer cells or immortalized cell lines.[22-24] Telomeric and centromeric probes are also applied to metaphase chromosomes (Fig. 8-20) to detect aneuploidy and structural abnormalities.

Preparation of chromosomes for metaphase FISH procedures begins with the culture of cells for 72 hours. About 45 minutes before harvesting, colcemid is added



■ Figure 8-19 Chromosome painting showing a derivative chromosome formed by movement of a fragment of chromosome 12 (black) to an unidentified chromosome.

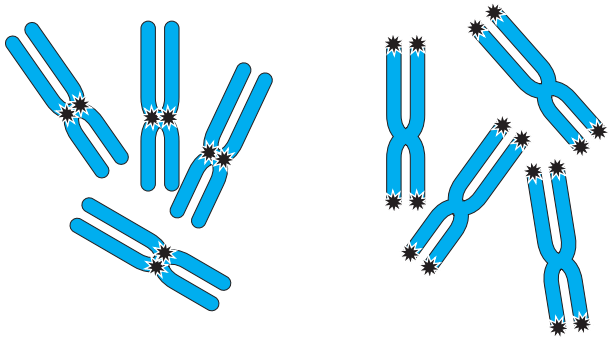


Figure 8-20 Centromeric (left) and telomeric (right) probes on metaphase chromosomes.

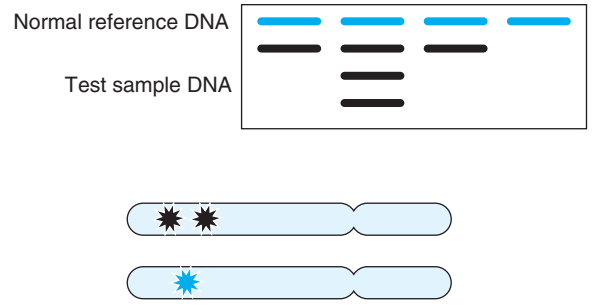


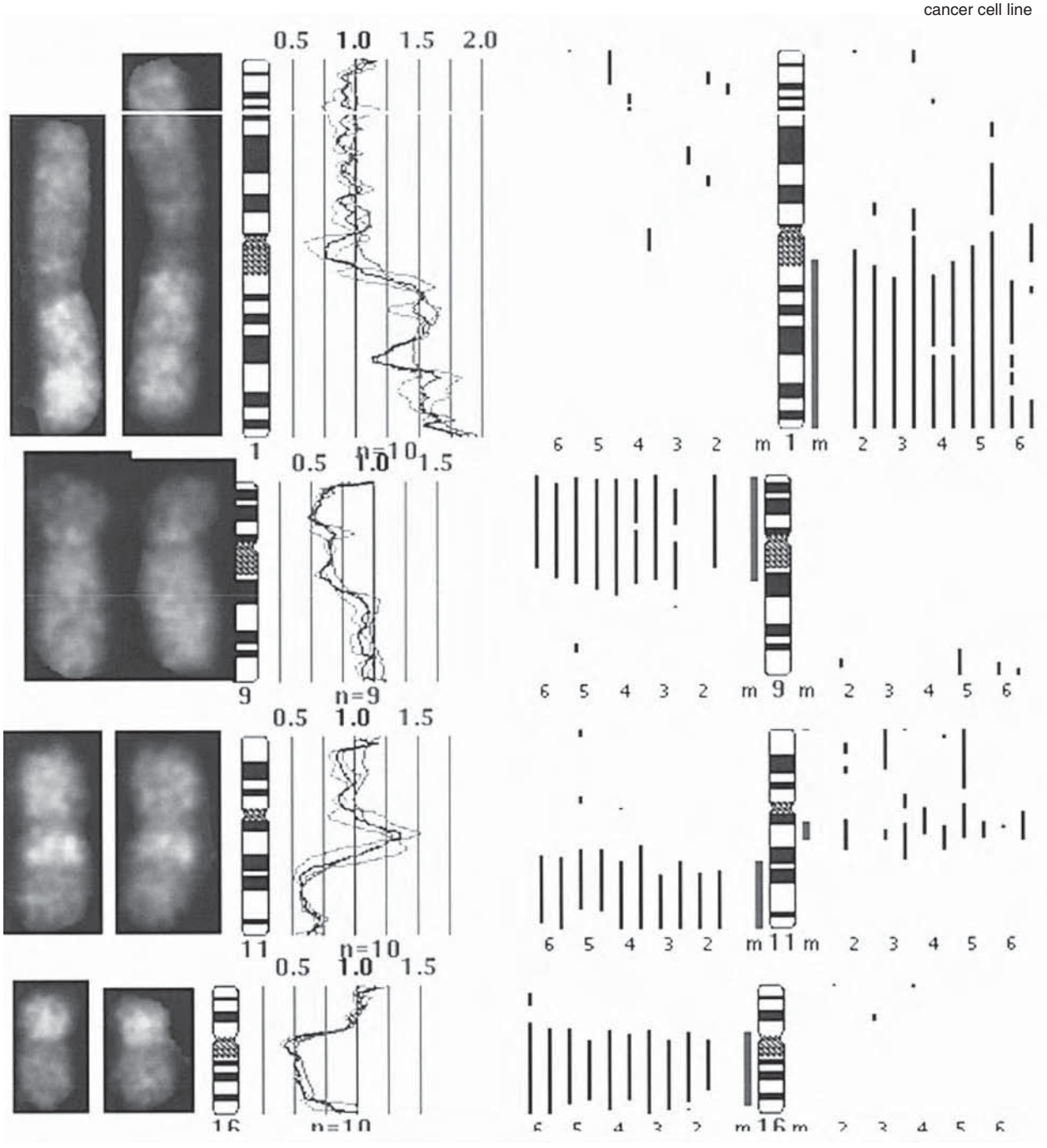
Figure 8-21 In CGH, the test sample is compared with a normal reference sample on a metaphase spread. Normally, test and reference signals are equal. A higher test signal denotes an amplification, and a higher reference signal denotes a deletion.

to the cultures to arrest cells in metaphase. The cells are then suspended in a hypotonic medium (0.075 M KCl) and fixed with methanol/acetic acid (3:1). The fixed-cell suspension is applied to an inclined slide and allowed to dry briefly. A second treatment with 70% acetic acid may improve the chromosome spreading and decrease background. Condensed chromosome spreads, especially those from cultured metaphases, may be affected by temperature and humidity. Under a phase contrast microscope, the chromosomes should appear well separated with sharp borders. Cytoplasm should not be visible. Once the slide is dried, hybridization proceeds as discussed above for interphase FISH.

Intrachromosomal amplifications or deletions can be detected by **comparative genome hybridization** (CGH).[25,26] In this method, DNA from test and reference samples is labeled and used as a probe on a normal metaphase chromosome spread (Fig. 8-21). CGH has the advantage of being able to identify the location of deletions or amplifications throughout the genome.[27] The resolution (precise identification of the amplified or deleted region), however, is not as high as can be achieved with **array CGH** (see Chapter 6).

For CGH, the test DNA is isolated and labeled along with a reference DNA. Cyanine dyes are used as fluorescent labels for test and reference DNA for CGH. The two colorimetrically distinct dyes, Cy3 and Cy5, are commonly used for this purpose. Cy3, which fluoresces at a wavelength of 550 nm, is often represented as "green," and Cy5, which fluoresces in the far-red region of the spectrum (650–667 nm), is represented as "red." Derivatives of these dyes, such as Cy3.5, which fluoresces in the red-orange region, are also available. Because these dyes fluoresce brightly and are water-soluble, they have been used extensively for CGH using imaging equipment.

Labeling (attachment of Cy3 or Cy5 dye to the test and reference DNA) is achieved by nick translation or primer extension in which nucleotides covalently attached to the dye molecules are incorporated into the DNA sequences. Dye-nucleotides commonly used for this type of labeling are 5-amino-propargyl-2′-deoxycytidine 5′-triphosphate coupled to the Cy3 or Cy5 fluorescent dye (Cy3-AP3-dCTP, Cy5-AP3-dCTP) or 5-amino-propargyl-2′-deoxyuridine 5′-triphosphate coupled to the Cy3 or Cy5 fluorescent dye (Cy3-AP3-dUTP, Cy5-AP3-dUTP). DNA to be tested is partially digested with DNase to produce fragments that will bind efficiently to the denatured DNA in a metaphase chromosome spread. Separate aliquots of test and reference DNA are labeled with different Cy3 and Cy5 dyes, respectively, before application to a normal metaphase spread. An example of results from a CGH analysis is shown in Figure 8-22. Despite its utility and versatility in detecting chromosomal abnormalities, CGH does require advanced technical expertise. Array CGH is less comprehensive, but more specific, for detection of particular abnormalities.



■ Figure 8-22 CGH analysis of four chromosomes from a cancer cell line. Amplified or deleted areas can be observed where the test and reference signals are not equal. The vertical lines on the diagram at right represent results from six different chromosomal spreads analyzed for excess reference signal (left of idiogram) or test signal (right of idiogram).

• STUDY QUESTIONS •

1. During interphase FISH analysis of a normal specimen for the t(9;22) translocation, one nucleus was observed with two normal signals (one red for chromosome 22 and one green for chromosome 9) and one composite red/green signal. Five hundred other nuclei were normal. What is one explanation for this observation?

2. Is 47; XYY a normal karyotype?

3. What are the genetic abnormalities of the following genotypes?
   47, XY, +18
   46, XY, del(16)p(14)
   iso(X)(q10)
   46,XX del(22)q(11.2)
   45, X

4. A chromosome with a centromere not located in the middle of the chromosome but not completely at the end, where one arm of the chromosome is longer than the other arm, is called:
   a. metacentric
   b. acrocentric
   c. paracentric
   d. telocentric

5. A small portion of chromosome 2 has been found on the end of chromosome 15, and a small portion of chromosome 15 has been found on the end of chromosome 2. This mutation is called a:
   a. reciprocal translocation
   b. inversion
   c. deletion
   d. robertsonian translocation

6. Phytohemagglutinin is added to a cell culture when preparing cells for karyotyping. The function of the phytohemagglutinin is to:
   a. arrest the cell in metaphase
   b. spread out the chromosomes
   c. fix the chromosomes on the slide
   d. stimulate mitosis in the cells

7. A CEP probe is use to visualize chromosome 21. Three fluorescent signals are observed in the patient's cells when stained with this probe. These results would be interpreted as consistent with:
   a. a normal karyotype
   b. Down's syndrome
   c. Klinefelter's syndrome
   d. technical error

8. Cells were harvested from a patient's blood, cultured to obtain chromosomes in metaphase, fixed onto a slide, treated with trypsin, and then stained with Giemsa. The resulting banding pattern is called:
   a. G banding
   b. Q banding
   c. R banding
   d. C banding

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Chapter 9

Lela Buckingham

Gene Mutations

OUTLINE

OBJECTIVES

- Compare phenotypic consequences of different types of point mutations.
- Distinguish detection of known mutations from scanning for unknown mutations.
- Discuss methods used to detect point mutations.
- Determine which detection methods are appropriate for screening of new mutations or detection of previously identified mutations.
- Describe mutation nomenclature for expressing sequence changes at the DNA, RNA, and protein levels.

Gene mutations include deletions, insertions, inversions, translocations, and other changes that can affect one base pair to hundreds or thousands of base pairs. Large differences in DNA sequence will likely have a significant effect on protein sequence. Alterations of a single or a few base pairs, or **point mutations**, will have a range of effects on protein sequence. Refer to the genetic code in Chapter 3, Figure 3-7, to see how a difference of one or a few base pairs may or may not change the amino acid designation.

Types of Gene Mutations

Because there is more than one codon for most of the amino acids, DNA sequence changes do not necessarily change amino acid sequence. This is an important concept for interpreting results of mutation analyses. Substitution of one nucleotide with a different nucleotide may be **silent**; that is, without changing the amino acid sequence (Table 9.1). **Conservative** substitutions may change the amino acid sequence, but the replacement and the original amino acid have similar biochemical properties, e.g., leucine for valine, and the change will not affect protein function significantly. In contrast, a **nonconservative** mutation is the substitution of a biochemically different amino acid, e.g., proline for glutamine, which changes the biochemical nature of the protein. A **nonsense** mutation terminates proteins prematurely when a nucleotide substitution produces a stop codon instead of an amino acid codon. Insertion or deletion of more or fewer than three nucleotides results in a **frameshift** mutation, throwing the triplet code out of frame. The amino acids in the chain after the frameshift mutation are affected, as the triplet code will include new combinations of three nucleotides. The genetic code is structured such that frameshifts often terminate protein synthesis prematurely because a stop codon appears sooner in the out-of-frame coding sequence than it would in a nonmutated reading frame.

Table 9.1 **Types of Point Mutations**

DNA Sequence	Amino Acid Sequence	Type of Mutation
ATG CAG GTG ACC TCA GTG	M Q V T S V	None
ATG CAG GTT ACC TCA GTG	M Q V T S V	Silent
ATG CAA GTG ACC TCA GTG	M Q L T S V	Conservative
ATG CCG GTG ACC TCA GTG	M P V T S V	Nonconservative
ATG CAG GTG ACC TGA GTG	M Q V T ter	Nonsense
ATG CAG GTG AAC CTC AGT G	M Q V N L S	Frameshift

Nonconservative, nonsense, and frameshift mutations generate a range of phenotypes, depending on where they occur along the protein sequence. Point mutations in the end of a coding region may have minimal consequences, whereas mutations at the beginning of a coding sequence are more likely to result in drastic alterations or even effective deletion of the protein coding region.

These factors are important when interpreting results of mutation analyses. Merely finding a difference between a test DNA sequence and a reference sequence does not guarantee an altered phenotype. Some screening methods designed to detect point mutations over large sequence regions do not determine the specific sequence alterations and, therefore, cannot distinguish among silent, conservative, and nonconservative changes. The specific type of mutation may be ascertained from a family history or determination of the specific sequence change with a second confirmatory test.

Advanced Concepts

The nature of the genetic code is such that frameshift mutations lead to a termination codon within a small number of codons. This characteristic might have evolved to protect cells from making long nonsense proteins.

Detection of Gene Mutations

Some, mostly inherited, disease-associated sequence changes in DNA occur frequently, e.g., for the factor V Leiden mutation and the hemochromatosis C282Y, H63D, and S65C mutations. Also, increasing numbers of specific single nucleotide polymorphisms (SNPs, see

Chapter 10) are being mapped close to disease genes. These changes, although outside of the disease gene, are detected as specific sequence changes frequently inherited along with the disease phenotype.

Some diseases are associated with many mutations in a single gene. For instance, there are more than 600 disease-associated mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene, and more than 2000 cancer susceptibility mutations have been reported in the *BRCA1* and *BRCA2* genes. Furthermore, unknown numbers of gene mutations are yet to be discovered. Detection of mutations in large genes requires screening across thousands of base pairs to detect a single altered nucleotide. To date, other than sequencing, there is no genome-wide scanning procedure that can identify yet unreported mutations.[1]

In molecular diagnostics, mutation detection is performed on a variety of specimen types. Inherited mutations are detected from the most convenient and noninvasive specimen material, such as blood or buccal cells. Somatic mutations are often more challenging to find because cells harboring mutations may be only a small fraction of the total specimen that consists of mostly normal cells. Under these circumstances, detection procedures must identify a single mutated gene from among thousands of normal genes. Polymerase chain reaction (PCR) amplification, which is part of many procedures, has simplified mutation detection, especially from limiting specimens. The use of PCR or other amplification methods to facilitate mutation detection must be performed under conditions that minimize the introduction of mutations in the course of amplification.

Interpretation of the results of mutation analyses is also challenging. Mutation scanning by methods that do not indicate the primary sequence change do not differentiate between silent, conservative, and nonconservative mutations. The actual effect on phenotype is left to post-test interpretation of supporting clinical data and patient family history. Mutations discovered through this type of scanning can be subjected to sequence analysis to confirm and further characterize the mutated region.

Although DNA sequencing is the most definitive method for detecting mutations (see Chapter 10), sequencing may not be appropriate, especially for high throughput procedures. A number of techniques have been designed for detection of DNA mutations from single base pair changes to large chromosomal rearrangements without having to determine the primary DNA sequence. Some of these methods are described below.

Sequence detection methods can be generally classified according to three broad approaches: **hybridization-based** methods, **sequence (polymerization)-based** methods, and **enzymatic** or **chemical cleavage** methods. Brief descriptions of representative methods are presented in the following sections. The methods selected are currently used or proposed for use in clinical applications. A summary of the methods discussed is shown in Table 9.2.

Hybridization-Based Methods

Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (**SSCP**)[2–4] is one of the more frequently used mutation screening procedures in the clinical laboratory. The method is based on the preference of DNA (as well as RNA) to exist in a double-stranded, rather than single-stranded, state. In the absence of a complementary strand, nucleic acids form intrastrand duplexes to attain as much of a double-stranded condition as possible. Each folded strand forms a three-dimensional structure, or **conformer**, the shape of which is determined by the primary sequence of the folded strand.

SSCP is determined by the migration of the single-stranded conformers in polyacrylamide gels under precisely controlled denaturing and temperature conditions. For SSCP, dilute concentrations of short, double-stranded PCR products, optimally 100–400 base pairs (bp) long, are denatured (e.g., in 10–20 mM NaOH, 80% formamide for 5 minutes at 95°C; or 10–20 mM NaOH, 0.004 mM EDTA, 10% formamide for 5 minutes at 55°–60°C) followed by rapid cooling. Because the diluted single strands cannot easily find their homologous partners under the concentration, buffer conditions, and temperatures used, they fold by intrastrand hybridization, forming three-dimensional conformers. The shape of the conformer depends on the complementary nucleotides available for hydrogen bonding and folding. A single bp difference in the DNA sequence can cause the conformer to fold differently. These conformers are resolved in a polyacrylamide gel or by capillary electrophoresis with temperature control.[5] The speed of migration depends on the shape as well as the size of the conformer. Differences

Table 9.2 Summary of Mutation Detection Methodologies

Method*	Target† (bp)	Accuracy‡ (%)	Specificity§ (%)	Sensitivity‖ (%)	Laboratory Application¶	Reference#
Sequencing	>1000	100	100	10–20	C, R	Chapter 9
SSCP	50–400	70–100	80–100	5–20	C, R	125, 126
DGGE	200–500	95–100	90–100	1–15	R	127, 128
TTGE	200–1000	95–100	90–100	1–10	R	129, 130
ASO	Defined	100	90–100	5–20	C, R	33, 131
HR-MCA	Defined	95–100	95–100	1–5	C, R	39, 41, 132
MIP	Defined, multiplex	100	95–100		R	46
HA, DHPLC	50–1000	95–100	85–100	5–20	C, R	50, 54, 55, 57, 133–138
Array Technology	Defined, multiplex	95–100	80–100	1–5	C, R	57, 59, 138
SSP	Defined	98–100	95–100	0.0005	C, R	74, 139
ddF	40–600	85–100	70–100	1–15	R	76–77
Allelic discrimination	Defined	95–100	90–100	0.0001	C,R	40, 68, 69
Dye terminator	Defined	98–100	95–100	5–10	R	81, 83, 140
PTT	500–2500	95–100	85–100	10–20	R	93, 141
PCR-RFLP	Defined	100	100	0.01–1	C, R	97
BESS	200–600				R	102, 142
NIRCA	500–1000	85–90	80–90	5–10	R	103, 143, 144
Invader	Defined, multiplex	100	95–100		C, R	112, 145
CCM	40–30,000	85–100		0.01–1	R	1

*See text. Data are from methods done under optimal conditions.
†Optimal length of sequence that can be screened accurately; defined methods target a single nucleotide or site; multiplex methods target multiple defined types in the same reaction.
‡Concordance with direct sequencing or other assays reported in the references.
§True positive detection of mutations without concurrent false-positive.
‖Detection of one mutant target in a background of normal targets.
¶C: Presently used in clinical applications; R: Research applications.
#Also see references in text.

in the shape of the conformers (kinks, loops, bubbles, and tails) are caused by sequence differences in the DNA single strand (Fig. 9-1). The band or peak patterns are detected by silver stain, radioactivity, or fluorescence. To avoid renaturation of homologous partners, a low concentration of products after denaturation must be maintained. As a consequence, less sensitive stains such as ethidium bromide are not often used for this assay. Band or peak patterns different from those of normal sequence control conformers prepared simultaneously with the test conformers indicate the presence of mutations.

SSCP is reported to detect 35%–100% of putative mutations.[6] The assay can be sensitive enough to detect mutations in samples containing as low as 5% potentially mutant cells,[7] although specimens that are at least 30% potentially mutant cells produce more reliable results. This requirement is satisfied in inherited mutations, as at least 50% of cells of a specimen will potentially carry a mutation. For somatic mutations, however, such as the analysis of tumor cells, the potentially mutant cells may be mixed with or surrounded by a vast majority of normal cells or tissue. Consequently, a cell suspension that is at

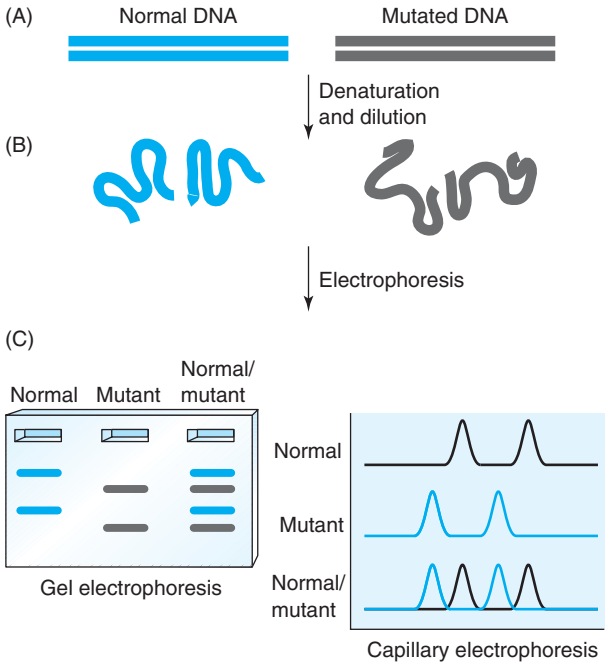


Figure 9-1 Single-strand conformation polymorphism analysis. Double-stranded PCR products (A) of normal or mutant sequences are denatured and form conformers (B) through intrastrand hydrogen bonding. These conformers can be resolved (C) by gel (left) or capillary (right) electrophoresis.

least 30% tumor cells or a microdissection of solid tumor tissue from fixed or frozen sections is recommended.

For microdissection of tissue sections, deparaffinized slides are stained with a mixture of 0.125% toluidine blue and 0.008% methylene blue and are examined by microscope. Areas containing tumor cells are identified based on morphology and selectively scraped from the slide or removed by extraction systems such as Pinpoint (Zymo Research). Laser capture microdissection instruments, capable of selecting and removing single cells, may also be used; however, most clinical laboratories do not have access to these instruments. The material removed from the sections is extracted at 50°-55°C in a lysis buffer of 10 mM Tris, 1.0 mM EDTA, 1 μg/μL proteinase K, or any of a number of lysis conditions that have been reported to produce lysates suitable for PCR. Because SSCP works more accurately in some genes than others, modifications of the SSCP procedure have been developed; for instance, using RNA instead of DNA (**RNA-SSCP** or **rSSCP**)[8,9] or using restriction endonuclease fingerprinting (**REF-SSCP**).[10] These latter methods, although more sensitive, are more difficult to interpret and not in general use.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (**DGGE**) exploits differences in denaturation between a normal and mutated DNA molecule caused by even one nucleotide difference in a sequence. The contribution of the attraction between successive bases on the same DNA strand (**stacking**) can affect denaturation of double-stranded DNA.[11–13] For DGGE, double-stranded DNA fragments 200–700 bp in length are prepared by PCR amplification of test sequences or by restriction digestion. The fragments are separated on polyacrylamide gels containing a gradient of concentrations of urea and formamide. A 100% denaturant solution is 7 M urea and 40% formamide. Gradients range 15%–90% denaturant, usually with a 10%–20% difference between the high denaturant concentration at the bottom of the gel and the low denaturant concentration at the top of the gel for a given analysis. Gradient gels can be prepared manually or with special equipment (gradient makers).

As the double-stranded DNA fragment moves through the gel, the denaturing conditions increase, sequences reach their denaturing point, and the complementary strands begin to denature. Domains of the sequences with different melt characteristics denature at different points in the gradient. The formation of single-stranded areas of the denaturing duplex slows migration of the fragment through the gel matrix from the point of the initial denaturation. Even a one-nucleotide difference between two DNA molecules results in the two molecules denaturing at different positions in the gel. The band of the mutated DNA shifts to a different position in the gel as compared with the normal DNA band. Complete strand separation is prevented by naturally occurring or artificially placed GC-rich sequences (**GC clamps**). These can be conveniently placed at the ends of PCR products by using primers tailed on the 5′ end with a 40 bp 5′ GC tail.

Two gradient orientations are used in DGGE. The gradient can increase horizontally across the gel; that is, perpendicularly to the direction of sample migration (**perpendicular DGGE**), or the gradient can increase vertically, parallel to the direction of sample migration (**parallel DGGE**; Fig. 9-2). In the former configuration, a mixture of samples is loaded across the entire gel in a single well, and a sigmoid curve of migration is observed, corresponding to the denaturing characteristics

Advanced Concepts

DGGE requires a significant amount of preparatory work to optimize conditions for detection of a particular gene mutation. Originally performed on restriction fragments, PCR products are now used for DGGE. Primers are chosen so that the region to be screened for mutations has one or two discrete melting domains (excluding the GC clamp) because more than two domains may give a complex pattern that is hard to interpret. The GC clamp should be positioned adjacent to the highest melting domain. Design of the primers and the melt characteristics of the resulting product require inspection of the sequence to be screened for mutations. The optimal gradient and gel running conditions must also be established. Initially, sample sequences are separated on a wide gradient (20–80% formamide) to find the area where the sequence migrations are most distinct. This area will define a narrower gradient (e.g. 30–55% gradient) for use in the actual test. The gel running conditions must be strictly controlled for reproducible results. If either run time or temperature, for instance, is not optimal, resolution of differing sequences may be lost.

of the sequences. This type of gradient is used to establish the more defined gradient conditions used in parallel DGGE. For parallel DGGE, a smaller gradient is used; samples are loaded in single lanes and analyzed by lane comparison. Because higher concentrations of DNA are used for this assay, detection with ethidium bromide is sufficient to visualize the results of the electrophoresis. Specific regions within large sequence areas may be visualized by blotting the bands in the DGGE gel to a nitrocellulose membrane and probing for the specific sequence (Southern blot). As with SSCP, DGGE gels are analyzed for banding patterns in the test specimens that differ from banding patterns of the control sequences.

DGGE has been used to detect tumor suppressor gene mutations,[14] clonality,[15] and population polymorphisms.[16] In **genomic DGGE**,[17] in which restriction fragments of genomic DNA rather than PCR products are separated on a gradient gel and then blotted and probed

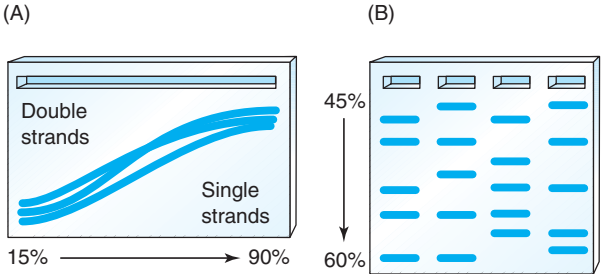


Figure 9-2 Schematic of perpendicular (A) DGGE and parallel (B) DGGE.

as a Southern blot, any area of the genome can be probed for mutations.

Two methods that are similar in design to DGGE are constant gradient gel electrophoresis (**CDGE**[18,19]) and temporal temperature gradient gel electrophoresis (**TTGE**[20,21]).

CDGE requires the initial determination of optimal denaturant concentrations for a particular target mutation. This can be ascertained by perpendicular DGGE or by using computer programs designed to predict the melting characteristics of a nucleotide sequence for a range of temperature and denaturing conditions. The sample is then run at the one optimal combination of denaturant concentration and temperature. As parameters must be set in this manner, CDGE is used for detecting known mutations rather than for screening for unknown mutations. CDGE has been extended to capillary electrophoresis (constant denaturant capillary electrophoresis), which increases the speed and resolution of the separation.[22] CDGE has been used to detect mutations in cancer genes.[23]

TTGE is similar to CDGE in that specific concentrations of formamide and urea are used to denature DNA duplexes. In TTGE, unlike CDGE, differences in denaturation are resolved by slowly raising the temperature of the gel during migration, e.g., 63°–68°C at 1.7°C/h. This

Advanced Concepts

Compared with SSCP, DGGE has less sensitivity for detecting mutations in genes that are rich in GC content.[146]

provides a wider range of denaturing conditions such that fragments requiring different denaturant compositions by CDGE can be resolved on a single gel by TTGE. This technique has been used in cancer,[24,25] genetic,[26,27] and industrial[28,29] applications.

Allele-Specific Oligomer Hybridization

Allele-specific hybridization, or **allele specific oligomer hybridization** (ASO), utilizes the differences in melting temperatures of short sequences of ~20 bases with one or two mismatches and those with no mismatches. At specific annealing temperatures and conditions (stringency), a single-stranded probe will not bind to a near complementary target sequence with one or two mismatched bases, whereas a probe perfectly complementary to the target sequence will bind. ASO is a **dot blot** method, similar to Southern blot using immobilized target and labeled probe in solution. It has been used to test for known, frequently occurring mutations; for example, in the *BRCA1* and *BRCA2* gene mutations frequently observed in inherited breast cancer[30] and the p16 gene mutations in familial melanoma.[31] The procedure begins with amplification of the gene region of interest by PCR. After the PCR product is spotted onto nitrocellulose or nylon membranes, the membranes are soaked in a high salt NaOH denaturation solution. The DNA on the membranes is neutralized with dilute acid and permanently affixed to the membrane by baking or ultraviolet crosslinking. Labeled probes matching the normal and mutated sequences are then hybridized to the membranes in separate reactions under specific stringency conditions (Fig. 9-3). Some protocols recommend addition of unlabeled probe directed at the nontarget sequence to the labeled targeted probe in order to increase binding specificity.[32] Hybridization can take 2–12 hours. Following hybridization, free probe is rinsed from the membrane, and probe signal is detected over the spots containing sequences matching that of the probe (Fig. 9-4). This method has been used in clinical testing for detection of specific mutations and polymorphisms and for typing of organisms. ASO is also routinely used in the clinical laboratory for tissue typing (sequence specific oligonucleotide probe hybridization; see Chapter 15).

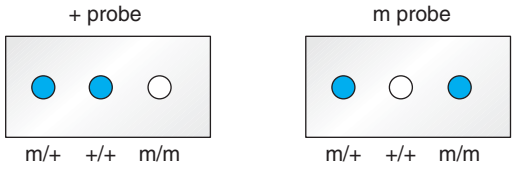


Figure 9-3 Allele-specific oligomer hybridization. Three samples are spotted on two membranes. One membrane is probed with a labeled oligomer of the normal sequence (+ probe, left) and the other with a labeled oligomer containing the mutation (m probe, right). A normal sample (+/+) hybridizes with the normal oligomer only. A homozygous mutant sample (m/m) hybridizes with the mutant oligomer only. A heterozygous mutant sample (m/+) hybridizes with both oligomers.

ASO analysis can also be carried out as a **reverse dot blot** in a 96 well plate format similar to capture probe methods developed for infectious disease testing, e.g., *Chlamydia trachomatis* (Amplicor CT/NG; Roche) and *Mycobacterium tuberculosis* (Amplicor MTB; Roche). For mutation analysis, mutant or normal probes are immobilized on the membrane. The sequence to be tested is amplified by PCR with one regular and one biotinylated

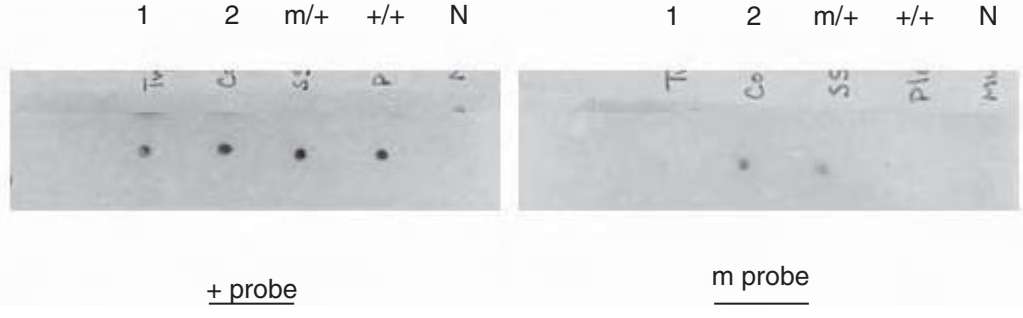


Figure 9-4 Autoradiography results of an allele-specific oligomer hybridization using chemiluminescent detection. One normal (1) sample and one heterozygous mutant (2) sample are shown with a heterozygous mutant control (m/+), a normal control (+/+), and a negative control (N).

primer. The biotinylated products are then exposed to the immobilized probes under conditions set so that only the exact complementary sequences hybridize. Unbound products are washed away, and those that remain bound are detected with a conjugated horseradish peroxidase-anti-biotin Fab fragment and exposure to chromogenic substrate. Generation of a color reaction indicates the binding of the test DNA to the normal or mutant probe. This method has been proposed for detection of frequently occurring mutations such as factor V Leiden.[33] HLA typing of multiple alleles on a single specimen is also performed by this method (see Chapter 15).

Melt Curve Analysis

Like DGGE and related methods, **melt curve analysis** (MCA) exploits the sequence- and stacking-directed denaturation characteristics of DNA duplexes.[34] The method is very useful as a postamplification step of real time PCR.[35,36] PCR amplicons generated in the presence of a DNA-specific fluorescent dye, such as ethidium bromide, SYBR Green, or LC Green, are heated at a rate of about 0.3°C/sec. The dyes, specific for double-stranded DNA, initially yield a high signal because the DNA is mostly double-stranded at the low temperature. As the temperature rises, the DNA duplexes begin to separate into single strands, losing dye accordingly. The fluorescent signal gives a pattern as shown in Figure 9-5. Sequence differences result in different melting characteristics and T_ms (where there are equal amounts of double- and single-stranded DNA) for each sequence. The T_m is often illustrated as a peak, plotting the derivative (speed of decrease) of fluorescence vs. temperature. Results are interpreted by the temperature peak placement with respect to the temperature on the *X* axis. Specimens with identical sequences should yield the same peak at the expected T_m, whereas specimens containing different sequences will yield two or more peaks (Fig. 9-6).

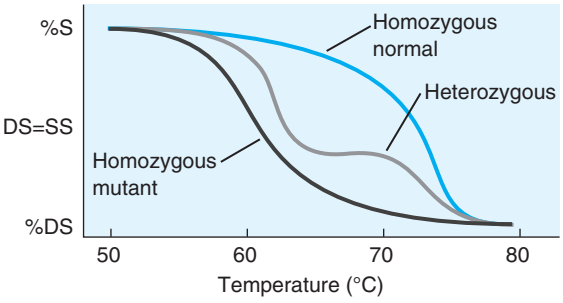


Figure 9-5 Melt curve analysis of homozygous mutant, heterozygous, and normal PCR products.

MCA of PCR products using nonspecific dyes is a simple and cost-effective way to screen for sequence differences. These dyes are not sequence-specific, however, and do not distinguish between the target amplicon and extraneous products in the PCR reaction, such as primer dimers or misprimed amplicons. Although the target sample should be identifiable by its T_m, such artifactual bands can complicate the melt curve and confuse interpretation. Specificity can be increased by using **high resolution melt curve analysis** (HR-MCA).[37–39] This method uses fluorescent resonance energy transfer (**FRET**) probes that hybridize next to one another across the sequence position being analyzed. The probes fluoresce only when bound to the target sequence because FRET fluorescence relies on the transfer of energy from a donor fluorescent molecule (fluor) on one probe to an acceptor fluor on the other probe. As the temperature increases, the probes dissociate at a specific T_m. When the probes dissociate from

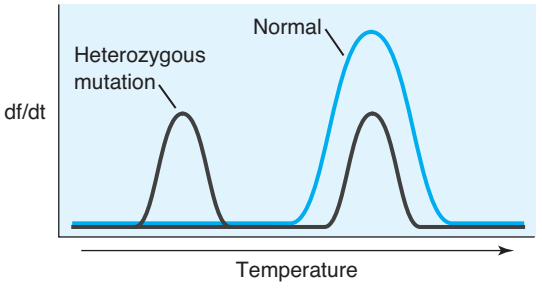


Figure 9-6 A plot of the derivative of the fluorescence data (df/dt) vs. temperature shows the inflexion point of the melt curve as a peak at the T_m of the test sequence. A normal homozygous sample should have a T_m that can be distinguished from that of the mutant sequence.

Advanced Concepts

PCR products smaller than 300 bp in size are preferred for melt curve analysis. The ability of the assay to distinguish sequence differences decreases with increasing size of the PCR product.[39]

the target, the donor is no longer close to the acceptor, and the fluorescence drops. If the target sequence has a mismatch between the target and the probe, hydrogen bonding is perturbed between the two strands of the double helix. The mismatch decreases the dissociation temperature, compared with matched or complementary sequences. A T_m lower than that of the probe and its perfect complement, therefore, indicates the presence of a mutation, or sequence difference between the known probe sequence and the test sequence.

FRET is most frequently performed with two probes; however, single-probe systems have been developed. The single probe is designed to fluoresce much more brightly when hybridized to the target. The fluorescence is lost on dissociation (Fig. 9-7). Another modification that is reported to improve the sensitivity of MCA is the covalent attachment of a minor groove binder (MGB) group to the probe. The MGB, dihydrocyclopyrroloindole tripeptide, folds into the minor groove of the duplex formed by hybridization of the terminal 5–6 bp of the probe with the template. This raises the melting temperature of the probe, especially one with high A/T content. The T_m of a 12–18 bp MGB conjugated probe is equivalent to that of a 25–27 bp non-MGB probe.[40]

Special instrumentation is required for MCA and HR-MCA. Thermal cyclers with fluorescent detection, such as the Roche LightCycler and the ABI 7000 series, have melt curve options that can be added to the thermal cycling program. The Roche LightTyper and the Idaho Technologies HR-1 systems are designed to do MCA only, but they can handle more samples per unit time than the thermal cycler systems.[41] Melt curve methodology has been proposed for a variety of clinical laboratory applications such as detection of DNA polymorphisms[42–44] and typing of microorganisms.[45]

Inversion Probe Assay

The molecular inversion probe system was designed as a method for detection of SNPs in DNA.[46] The molecular inversion probe is a linear probe containing two target-

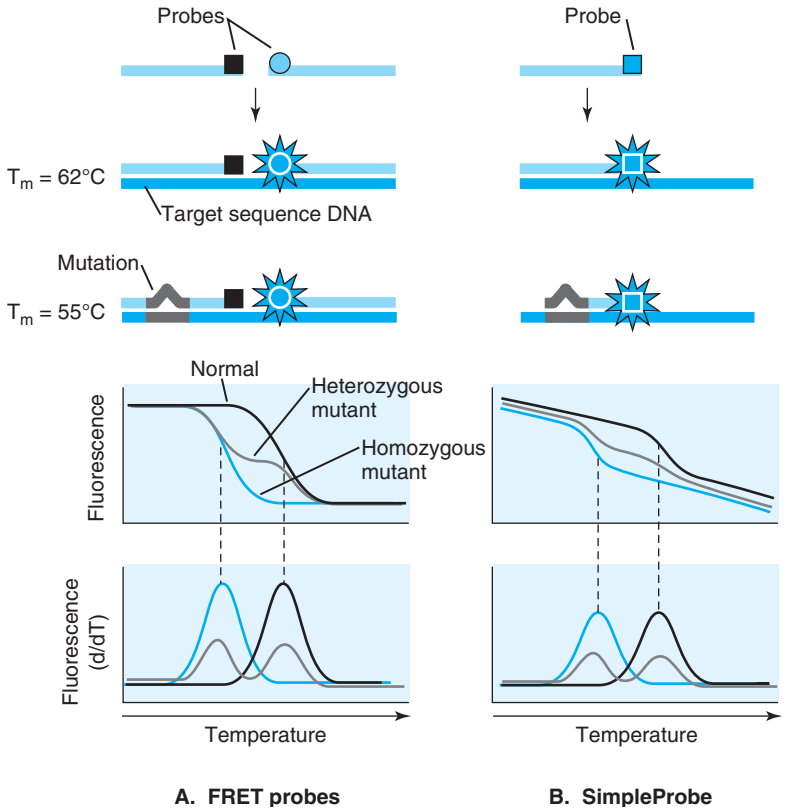


Figure 9-7 Melt curve analysis with FRET probes (left) and SimpleProbe (right). A mismatch between the target and probe will lower the T_m of the duplex.

specific regions, one at each end; primer binding sites; and a 20 nucleotide–long unique sequence tag (Fig. 9-8). The probe hybridizes to the target sequence, the two ends flanking the potential SNP being tested. In four separate reactions A, C, T, or G is added along with DNA polymerase and DNA ligase to the probe-target hybridization reaction (Fig. 9-9). A one-base extension and ligation of the probe occur only in the tube containing the nucleotide complementary to the SNP site on the template. Once the probe is ligated and circularized with a single-stranded endonuclease, the probe, which is then released from the target, inverts. The inverted probe is then amplified using fluorescently labeled primers complementary to primer binding sites at either end of the probe. The resulting amplicons from each tube are hybridized to one of four microarrays, probing for the unique sequence tag that identifies the genomic location of the mutation. Fluorescence will emit from the ligated and amplified probe bound to one of the arrays, i.e., the one corresponding to the nucleotide added to the probe.

The inversion probe assay is capable of screening multiple mutations or polymorphisms simultaneously as a **multiplex inversion probe** assay.[47] Because each probe has a unique sequence tag to identify it on the array step and common PCR primer sites, thousands of probes can be added to a single set of four reactions with genomic DNA (500 ng) in four wells of a 96 well plate. Each of the probes will be successfully ligated in one of the four nucleotide-extension reactions. As the PCR primer sites are the same for all probes, one set of primers can amplify

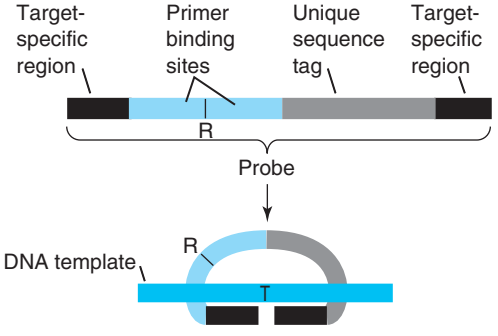


Figure 9-8 The molecular inversion probe is designed to recognize specific genomic targets on the template, as shown in the bottom panel. A restriction site R is for release of the probe after template-dependent circularization. A unique sequence tag identifies that target by its location of hybridization on a microarray.

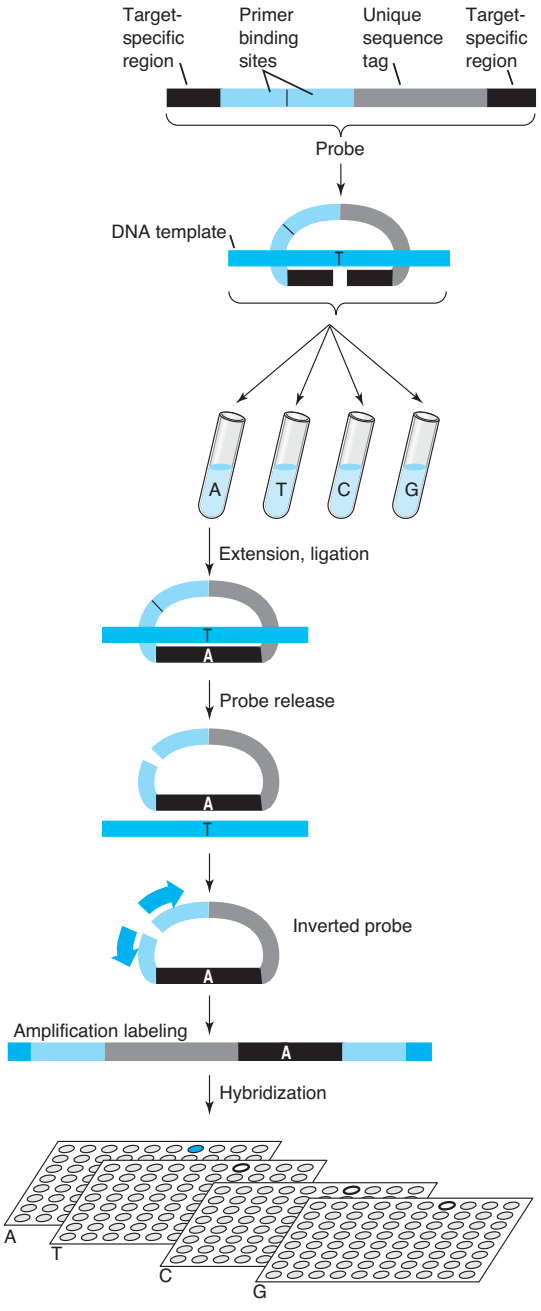


Figure 9-9 Molecular inversion probe procedure (only one of four reactions shown). Closure of the hybridized circular probe occurs only in the presence of the nucleotide complementary to the template. The circular probe from each tube is released, amplified, and labeled for hybridization to one or four arrays. Each probe hybridizes to one of the four arrays, depending on the original template sequence.

all ligated probes. The amplicons are then hybridized to microarrays by the unique sequence tags, which identify their genomic locations. The original multiplex inversion probe assay method used four separate identical microarrays, one for the amplified products of each extension reaction. Four-color dye technology now permits hybridization of all four reactions on the same microarray. The location of the array position identifies the location of the mutation. The color identifies the nucleotide at that location. This assay is one of the high throughput methods used in the Human Haplotype Mapping Project (see Chapter 11). Although the results of the project will provide targets for clinical laboratory testing, inversion probe assays are not to date directly used for clinical analysis.

Heteroduplex Analysis

Solution hybridization and electrophoresis of test amplicons mixed with reference amplicons can reveal mutations. To form **heteroduplexes**, nonidentical double-stranded DNA duplexes are heated to a temperature that results in complete denaturation of the double-stranded DNA (e.g., 95°C) and then slowly cooled (e.g., −1°C/4–20 sec). Heteroduplexes are formed when single strands that are not completely complementary hybridize to one another. (Heteroduplexes are also formed when test amplicons from genetically heterozygous specimens are denatured and renatured.) The heteroduplexes migrate differently than do homoduplexes through polyacrylamide or agarose gels (Fig. 9-10). The presence of bands different from a homozygous reference control is indicative of mutations. Gel-based heteroduplex methods have been designed for HIV typing[48] and hematological testing.[49]

Conformation-sensitive gel electrophoresis is a heteroduplex analysis method in which the heteroduplexes are resolved on a 1,4-bis (acrolyl) piperazine gel with ethylene glycol and formamide as mildly denaturing solvents to optimize conformational differences.[50,51] This method was intended for screening large genes for mutations and polymorphisms. The use of fluorescent detection increases sensitivity and throughput of the assay.

Heteroduplexes are also resolved by denaturing high-performance liquid chromatography (**DHPLC**). This version of heteroduplex analysis is performed on PCR products, ideally 150–450 bp in length. The amino acid analog betaine is sometimes added to the heteroduplex

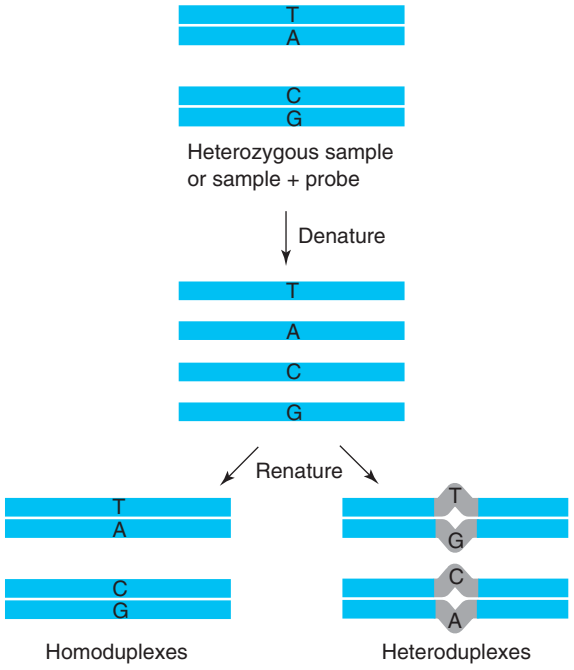


Figure 9-10 Heteroduplex analysis is performed by mixing sample amplicons with a reference amplicon, denaturing, and slowly renaturing. If the sample contains mutant sequences, a fraction of the renatured products will be heteroduplexes. These structures can be resolved from homoduplexes by electrophoresis.

mixture to minimize the differences in stability of AT and GC base pairs, increasing the sensitivity of detection.[52] HPLC separation is then performed on a 25%–65% gradient of acetonitrile in triethylammonium acetate at the melting temperature of the PCR product. The heteroduplexes elute ahead of the homoduplexes as the denaturing conditions intensify. The migrating homoduplexes and heteroduplexes are detected by absorbance at 260 nm or by fluorescence. HPLC methods are reported to be more sensitive than gel methods, with greater capacity for screening large numbers of samples.[53–55] Although gel-based heteroduplex analyses are routinely used in the clinical laboratory, HPLC analysis of heteroduplexes is still being evaluated as a mutation screening method in the clinical laboratory.[56]

Array Technology

Single base-pair resolution by hybridization differences is achievable with **high density oligonucleotide arrays**

and **microelectronic arrays** (see Chapter 6). These methods are similar to comparative genome hybridization as described in Chapter 8 but focus on a single gene with higher resolution as in ASO procedures. Mutation analysis of the p53 tumor suppressor gene by array analysis has sensitivity and specificity similar to that of direct sequencing.[57] The advantage of array methods is the large number of inquiries (potential sequence mutations or SNPs) that can be tested simultaneously.

Arrays can also be designed to test multiple genes for sequence mutations. To do this type of analysis, the test PCR-amplified DNA must be fragmented by treatment with DNase before binding to the complementary probes on the array. If the sample fragments are too large (not treated with DNase), a single base-pair mismatch has minimal effect on hybridization so that the fragment binds to multiple probes, and the specificity of detection is lost. An example of one type of hybridization format, **standard tiling**, is shown in Figure 9-11.[57] In this format, the base substitution in the immobilized probe is always in the twelfth position from its 3′ end. Commonly occurring mutations can be targeted in another type of format, **redundant tiling**, in which the same mutation is placed at different positions in the probe (at the 5′ end, in the middle, or at the 3′ end). After hybridization of the sample DNA, fluorescent label introduced during PCR amplification is read on a scanner with appropriate software to correct for background and normalized and the mutations are identified as indicated by which probes are bound. Although not performed routinely in clinical laboratories, a number of applied methods have been developed using high density oligonucleotide and microelectronic arrays.[58–60]

Bead array technology utilizes sets of color-coded polystyrene beads in suspension as the solid matrix. In an extension of the FlowMetrix system,[61] 100 sets of beads are dyed with distinct fluorochrome mixes. Each set is coated with oligonucleotide probes corresponding to a genetic locus or gene region. In this technology 10^5 or more probes are attached to each 3–6-micron bead. When labeled test samples are hybridized to the beads through complementary probe sequences, the combination of bead color and test label reveals the presence or absence of a mutation or polymorphism. The advantage of this arrangement is that multiple loci can be tested simultaneously from small samples. Up to 100 analytes can be

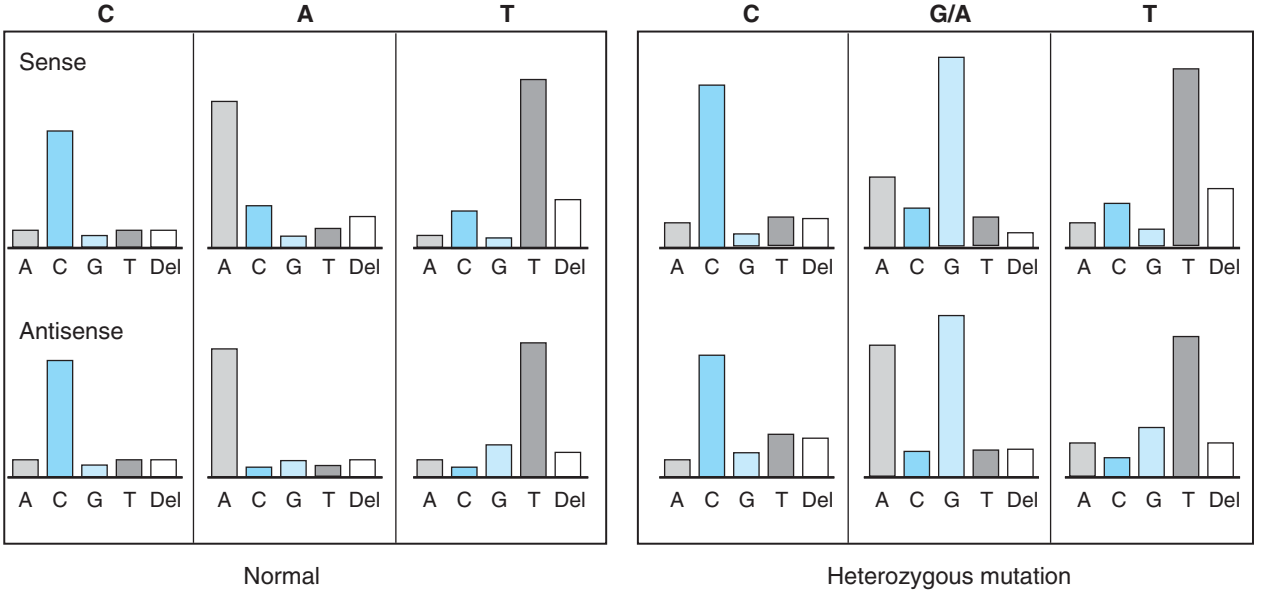


Figure 9-11 Mutation analysis of the p53 gene by high-density oligonucleotide array analysis. Each sequence position is represented by 10 spots on the array, 5 sense and 5 antisense probes. The sequence binds only to its exactly complementary probe. The illustration shows three adjacent sequence positions, CAT. Binding of the sample fragment is detected by increased fluorescence. A fragment with the normal sequence is on the right; a heterozygous mutation is on the left.

tested in a single well of a microtiter plate. This method requires a flow cytometry instrument, Luminex, that excites and reads the emitted fluorescence as the beads flow past a detector. This technology has been applied to antibody detection and infectious diseases and is used in tissue typing and in other clinical applications.[62–64]

Sequencing (Polymerization)-Based Methods

Sequence-Specific PCR

Sequence-specific PCR (**SSP-PCR**) is commonly used to detect point mutations and other single nucleotide polymorphisms. There are numerous modifications to the method, which involves careful design of primers such that the primer 3′ end falls on the nucleotide to be analyzed. Unlike the 5′ end, the 3′ end of a primer must match the template perfectly to be extended by *Taq* polymerase (Fig. 9-12). By designing primers to end on a mutation, the presence or absence of product can be interpreted as the presence or absence of the mutation.

Normal and mutant sequences can be analyzed simultaneously by making one primer longer than the other, resulting in differently sized products, depending on the sequence of the template (Fig. 9-13). Alternatively, primers can be multiplexed[30] (Fig. 9-14). Multiplexed SSP-PCR was originally called **amplification refractory mutation system PCR** or **tetra primer PCR.**[65,66] Sequence-specific PCR is routinely used for high resolution HLA typing (Chapter 15) and for detection of commonly occurring mutations.

A high throughput application of bead array technology (Illumina bead array[67]) uses sequence-specific PCR (Fig. 9-15). In this assay, tailed primers are used to amplify the test DNA. The resulting PCR products will have an allele-specific sequence at one end and a locus-specific sequence at the other end. This PCR product is subsequently amplified in a second round using Cy3 or Cy5 (fluorescently) labeled 5′ primers, corresponding to the normal or mutant allele and a common 3′ primer. These amplicons can then be hybridized to the beads. The bead color (locus) combined with Cy3 or Cy5 fluorescence (allele) types the allele at each locus. Although this system is one of the technologies used in the Human Haplotype Mapping Project, it is not routinely used in the clinical laboratory.

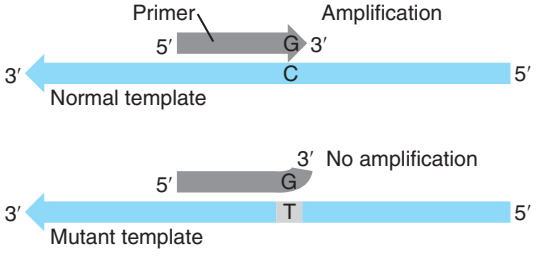


Figure 9-12 Sequence-specific primer amplification. Successful amplification will occur only if the 3′ end of the primer matches the template.

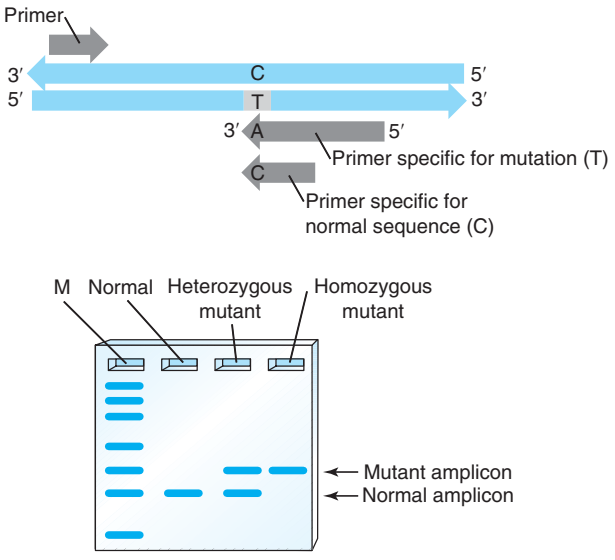


Figure 9-13 Allele-specific primer amplification of a C→T mutation. A longer primer is designed with the mutated nucleotide (A) at the 3′end. This primer is longer and gives a larger amplicon than the primer binding to the normal sequence (top). The resulting products can be distinguished by their size on an agarose gel (bottom). First lane: molecular weight marker; second lane: a normal sample; third lane: a heterozygous mutant sample; fourth lane: a homozygous mutant.

Allelic Discrimination With Fluorogenic Probes

Thermal cyclers with fluorescent detection support **allelic discrimination** with fluorogenic probes. This method is an extension of the 5′ nuclease PCR assay using two probes labeled with different fluors (Fig. 9-16). Each probe matches either the normal or mutant sequence. If

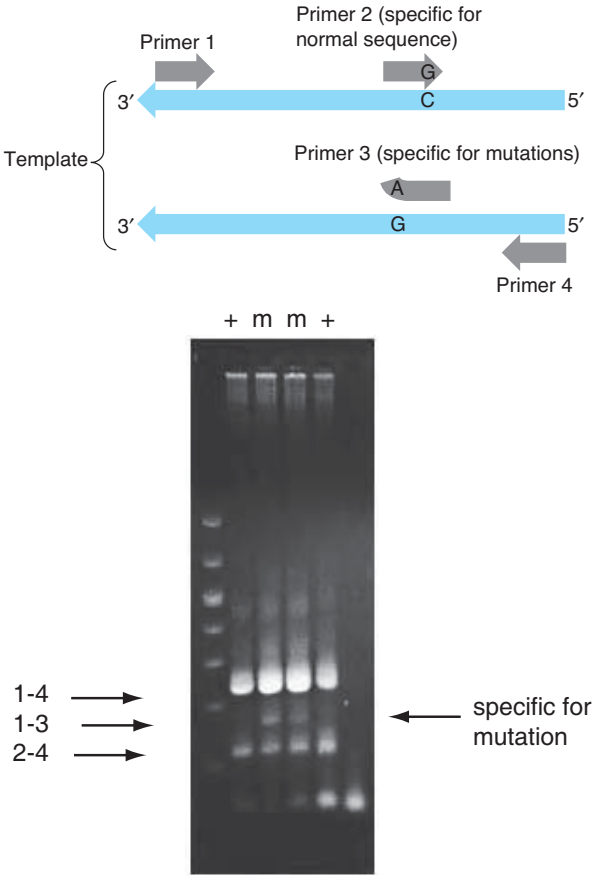


Figure 9-14 Multiplex allele-specific PCR. The mutation (C→A) is detected by an allele-specific primer (3) that ends at the mutation. Primers 3 and 4 would then produce a mid-sized fragment (1–3). If there is no mutation, a normal primer (2) binds and produces a smaller fragment (2–4). Primers 1 and 4 always amplify the entire region (1–4).

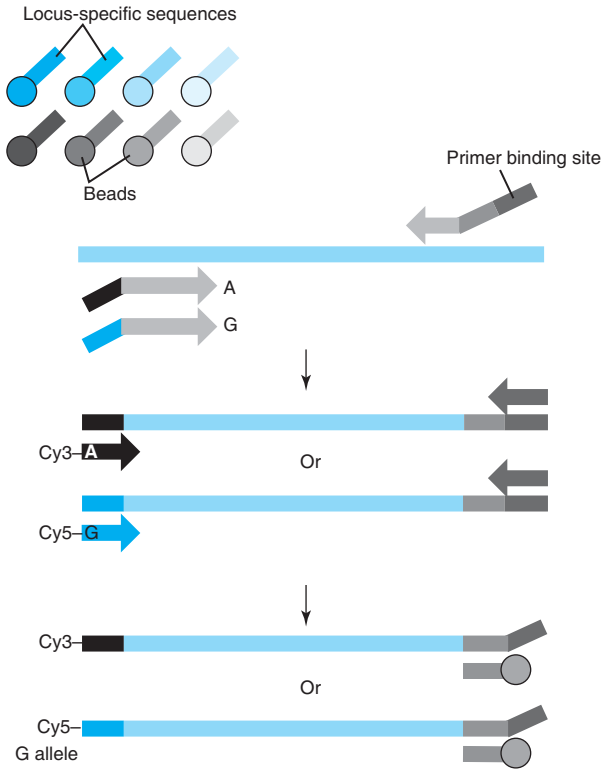
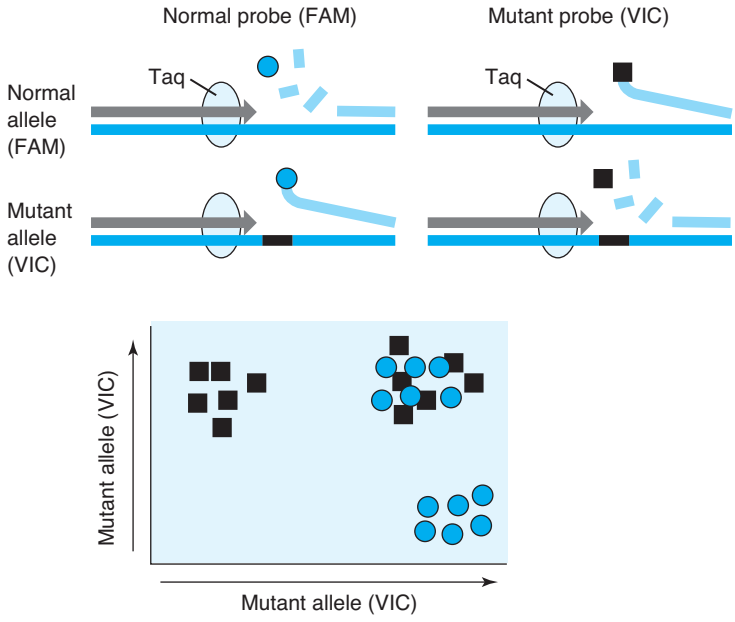


Figure 9-15 Bead array technology. Beads colored with distinct fluorescent dyes (upper left) are covalently attached to the probe sequences, each color of bead attached to a probe representing a specific locus. In a sequence-specific PCR, test DNA is amplified with tailed primers. The tailed PCR products are amplified in a second reaction to generate labeled amplicons that will bind to specific beads, according to the gene locus. The combination of bead label and the hybridized amplicon label reveals whether there is a mutant or normal allele at that locus.

either probe matches the test sequence, it is digested by the enzyme, releasing the reporter dye. The presence of the corresponding fluorescent signals indicates whether the test sequence is normal or mutant; that is, whether the probe matched and hybridized to the test sequence. In the example shown in Figure 9-16, the probe complementary to the normal sequence is labeled with FAM dye. The probe complementary to the mutant sequence is labeled with VIC dye. If the test sequence is normal, FAM fluorescence will be high, and VIC fluorescence will be low. If the test sequence is mutant, VIC will be high, and FAM will be low. If the sequence is heterozygous, both VIC and FAM will be high. Negative controls show no VIC or no FAM. This assay has the advantage of interrogating multiple samples simultaneously and has been proposed as a practical high throughput laboratory method.[68,69] It has been used in research applications in genetics and infectious disease.[70-74]

Dideoxy DNA Fingerprinting

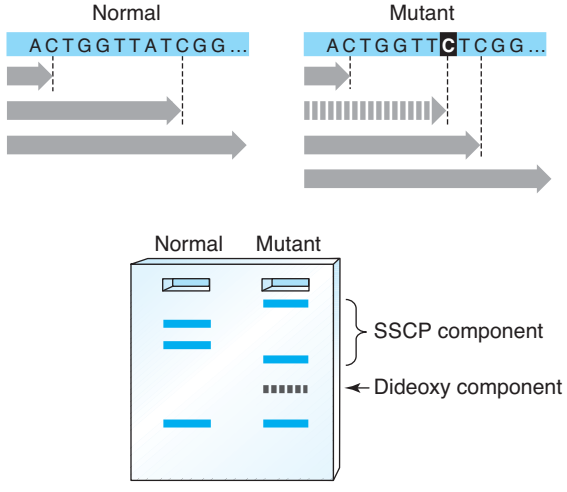
Dideoxy DNA fingerprinting (**ddF**) is a modified chain termination sequencing procedure (see Chapter 10 for a description of dideoxy chain termination sequencing). For this analysis, a single dideoxynucleotide is used to



■ Figure 9-16 Allelic discrimination. Probes, complementary to either the normal sequence (left) or the mutant sequence (right), are labeled with different fluors, e.g., FAM and VIC, respectively. The *Taq* exonuclease functions only if the probe is matched to the sequence being tested. High FAM indicates normal sequence, and high VIC indicates mutant sequence. If both fluors are detected, the test sample is heterozygous.

generate a series of terminated fragments that are resolved in one lane of a nondenaturing polyacrylamide gel. A combination of dideoxy sequencing, SSCP, and ddF resolves normal and mutant sequences by nucleotide base differences that result in the absence of a normal band or presence of an additional band (informative dideoxy component) and altered mobility of terminated fragments (informative SSCP component).

Dideoxynucleotide triphosphates (ddNTP) terminate DNA synthesis (see Chapter 10). For example, dideoxy guanosine triphosphate added to a DNA synthesis reaction terminates copying of the template at each C residue on the template. If the template sequence has a mutation that replaces another nucleotide with C (or substitutes a C residue with another nucleotide), an additional fragment terminated by ddG will be present on the gel, as compared with the normal pattern (Fig. 9-17). Furthermore, fragments terminated at C residues beyond the mutation migrate with altered mobility due to the base change. Whereas the additional fragment is absolute, the altered mobility is subject to gel conditions and temperature, just as with SSCP. Each of the four dideoxynucleotides can be used in this assay, depending on the nature of the sequence changes under study. This assay was introduced



■ Figure 9-17 Dideoxy fingerprinting detection of an A>C mutation. ddG added to the synthesis reaction terminates copies of the template at each opportunity to add G (opposite C in the template). An additional termination product will be generated from the mutant template (dotted line). This fragment will be detected as an extra band on the gel (dideoxy component). The subsequent larger terminated fragments will have a 1 bp difference from the normal ones, which may affect migration (SSCP component).

as an improvement in sensitivity over SSCP and other screening methods.[75]

The basic ddF procedure screens only one strand of the template duplex. By adding two primers instead of one, simultaneous termination reactions on both strands are generated in **bidirectional dideoxy fingerprinting.**[76] Although the band patterns produced by this method are more complex, mutations that might be missed on one strand will be detected in the complementary strand. Both ddF and bi-ddF can be performed using capillary electrophoresis as well as gel electrophoresis.[77,78] Although the methods are widely used in research applications, the extensive optimization required for consistent results has precluded their general use in the clinical laboratory.

Dye Terminator Incorporation

Multiplex assays have been designed using limited incorporation of fluorescently labeled dideoxynucleotide triphosphates. In one method, **fluorescent polarization-template–directed dye terminator incorporation** (FP-TDI)[79] primers are designed to hybridize on the test sequence up to the nucleotide being tested (Fig. 9-18). Two fluorescence-labeled terminator dideoxynucleotides corresponding to the alleles to be typed serve as primers for a single base extension reaction. For example, one nucleotide is labeled with the dye ROX, and the other is labeled with the dye BFL, as in the figure, ROX-ddTTP and BFL-ddCTP. The remaining two terminators, ddATP and ddGTP, are also present, although in unlabeled form, to prevent misincorporations. No deoxy nucleotides (dNTPs) are present. If a labeled ddNTP is incorporated onto the primer, the fluorescence polarization of that dye increases as it becomes part of the larger oligonucleotide.[80] The samples are read twice on a fluorometer, with filters corresponding to each of the dyes used. Instrument software calculates the polarization from the raw data and produces a numerical report. Although this procedure is compatible with mutation detection, its present use is in single nucleotide polymorphism analysis.[81]

Another extension/termination assay is **Homogeneous MassExtend** or **MassArray (SEQUENOM).**[82,83] In this method, mass spectrometry is used to detect extension products terminated by specific dye-labeled dideoxynucleotides. An example is shown in Figure 9-19. All four deoxynucleotides and one dideoxynucleotide, e.g., ddT, are added to the extension reaction. Depending on the allele in the test sequence, A or C in the example, the

Figure 9-18 FP-TDI detection of an A or G allele of a gene sequence. The color of the polarized fluorescence detected indicates which dideoxynucleotide is incorporated and, therefore, the nucleotide on the test template.

primer will either incorporate ddTTP and terminate at the A or continue to the next A in the sequence, producing a larger extension product. The products are then analyzed by mass spectrometry to distinguish their sizes. Like FP-TDI, this method can detect large numbers of mutations or polymorphisms simultaneously. The system does, however, require expensive instrumentation. Because of their high throughput detection capabilities, both FP-TDI and MassExtend were used in the Human Haplotype Mapping Project. Mutation detection by mass spectrometry may become more practical in the clinical laboratory as new methods are developed.[84–87]

Protein Truncation Test

Nonsense or frameshift mutations cause premature truncation of proteins. The **protein truncation test** (also called **in vitro synthesized protein** or **in vitro transcription/translation**) is designed to detect truncated

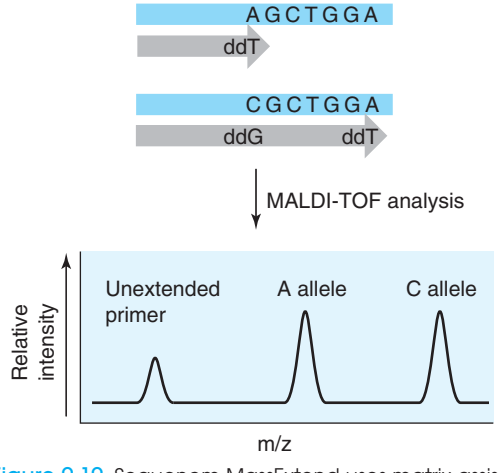


Figure 9-19 Sequenom MassExtend uses matrix assisted desorption/ionization-time of flight mass spectrometry to detect extension products of different sizes (mass).

proteins as an indication of the presence of DNA mutations.[88] This procedure uses a PCR product containing the area of the gene likely to have a truncating DNA mutation. The PCR product is transcribed and translated in vitro using commercially available coupled transcription/translation systems. When the peptide products of the reaction are resolved by polyacrylamide gel electrophoresis, bands below the normal control bands, representing truncated translation products, are indicative of the presence of DNA mutations (Fig. 9-20). This procedure has been used to detect mutations associated with breast cancer,[89,90] cystic fibrosis,[91] familial adenosis polyposis,[92] retinoblastoma,[93] and many other disease conditions. It has had limited use, however, as a clinical test.

Cleavage Methods

Restriction Fragment Length Polymorphisms

If a mutation changes the structure of a restriction enzyme target site or changes the size of a fragment generated by a restriction enzyme, restriction fragment length polymorphism (RFLP) analysis can be used to detect the sequence alteration. Analysis of RFLPs in genomic DNA by Southern blot is described in Chapter 6. To perform **PCR-RFLP**, the region surrounding the mutation is amplified, and the mutation is detected by cutting the amplicon with the appropriate restriction enzyme (Fig. 9-21). Mutations can inactivate a naturally occurring restriction site or generate a new restriction site so that digestion of the PCR product results in cutting of the mutant amplicon but not the normal control amplicon or vice versa. Although straightforward, PCR-RFLP requires careful design, as rare polymorphisms have been reported to confound RFLP results.[94] Several PCR-RFLP methods are widely used for detection of commonly occurring mutations, such as factor V Leiden[95] and HFE mutations. PCR-RFLP has also been used for HLA typing (see Chapter 15).

PCR-RFLP can be multiplexed to detect more than one gene mutation simultaneously. This has been practical for detection of separate gene mutations that affect the same phenotype, e.g., factor V Leiden and prothrombin.[96] Alternatively, a combination of SSP-PCR and PCR-RFLP is also applied to simultaneous detection of mutations in more than one locus. An example is shown in Figure 9-22, in which a primer designed to produce a restriction site in the amplicon is used for each gene in a multiplex PCR. In the example, the primers are designed to generate a *HindIII* site in the amplicons. The PCR reaction and the

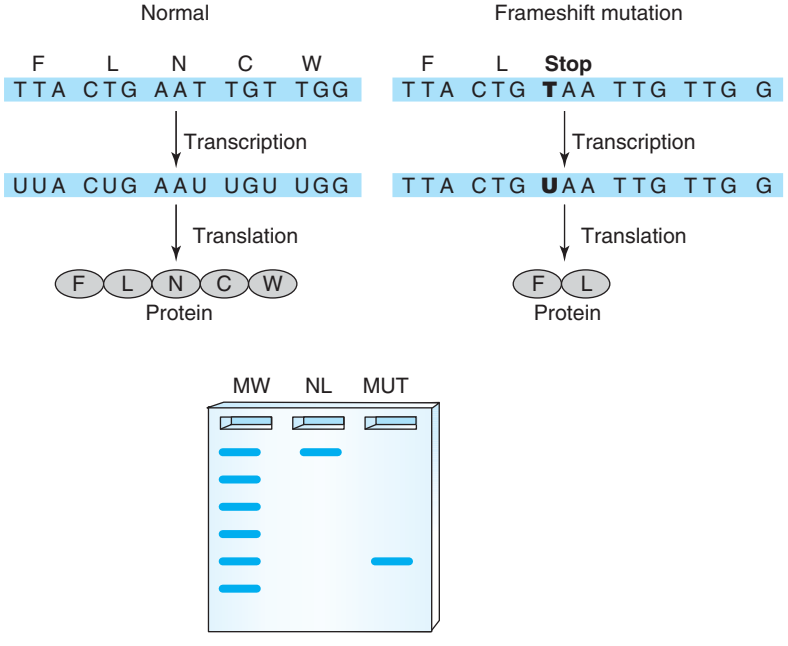


Figure 9-20 A frameshift mutation (top right) results in a truncated protein synthesized in vitro from a PCR product. The truncated peptide is resolved by polyacrylamide gel electrophoresis (bottom). Results from analysis of a homozygous mutant sample (MUT) and a normal sample (NL) are shown.

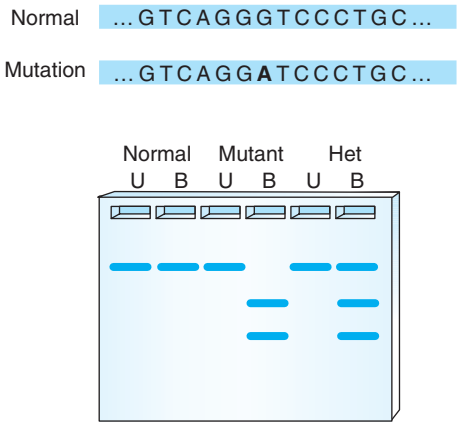


Figure 9-21 PCR-RFLP. The normal sequence (top line) is converted to a *Bam*H1 restriction site (GGATCC) by a G>A mutation. The presence of the mutation is detected by testing the PCR product with *Bam*H1. The bottom panel shows the predicted gel patterns for the homozygous normal, homozygous mutant, and heterozygous samples uncut (U) or cut with *Bam*H1 (B).

*Hind*III digestion are performed in the same tube, and the products are separated on one lane of the gel.[97-99] This procedure is used in clinical analysis of factor V Leiden and prothrombin mutations.

Heteroduplex Analysis With Single-Strand Specific Nucleases

The detection sensitivity of heteroduplex analysis can be increased by using single-strand–specific nucleases, e.g., S1 nuclease, that cleave heteroduplexes at the mispaired bases.[100] PCR amplifications and heteroduplex formation were described in the earlier section on heteroduplex analysis. After cooling, the heteroduplexes are digested with a single-strand–specific nuclease. Digested heteroduplexes (but not homoduplexes) yield smaller bands that can be resolved on an agarose gel (Fig. 9-23). In addition to detecting mutations, the fragment sizes can be used to estimate the placement of the mutation within the amplified sequence.

Base Excision Sequence Scanning

Base excision sequence scanning (**BESS**) is a PCR amplification in the presence of small amounts of deoxyuridine triphosphate (dUTP) added to the reaction mix, followed by treatment with excision enzymes that cleave the frag-

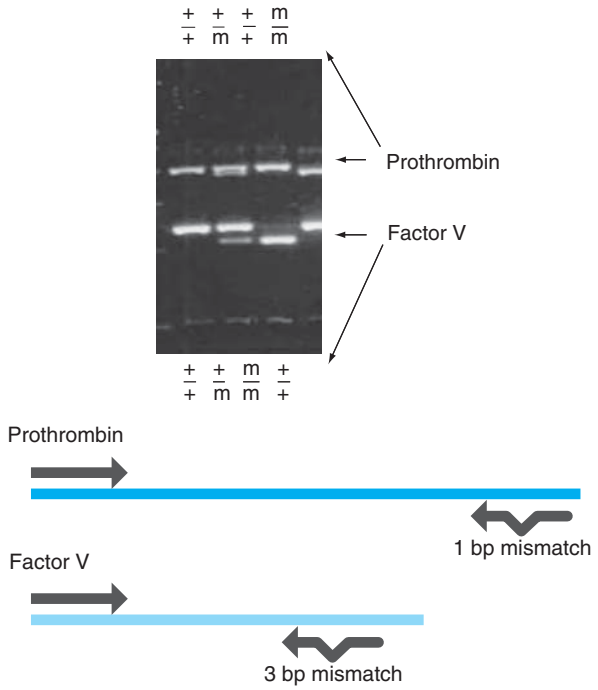


Figure 9-22 Multiplex PCR with mutagenic primers to detect mutations in factor V and prothrombin. The primer sequences are designed to generate a *Hind*III site in the PCR product if the mutations are present. The prothrombin and factor V PCR products are different sizes that can be resolved on the gel in a single lane.

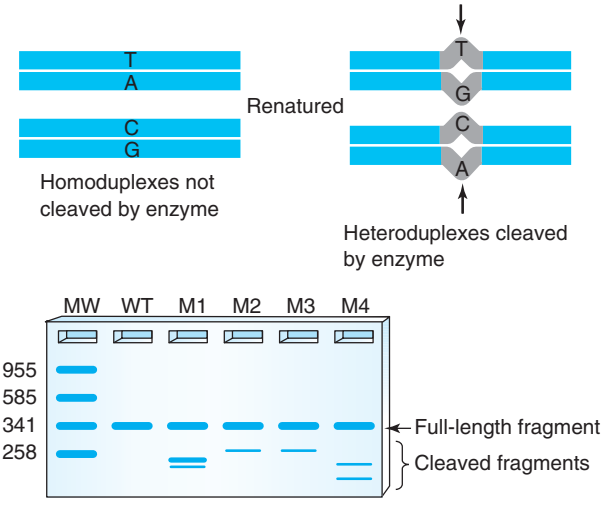


Figure 9-23 Single-stranded endonucleases cleave mispaired regions of heteroduplexes (top). The cleaved fragments can be resolved by agarose gel electrophoresis (bottom).

ment at the dU sites.[101] For example, the sequence to be scanned is amplified in a standard PCR reaction containing a mixture of 0.2 mM dNTPs and 0.015 mM dUTP. One of the primers in the PCR reaction has a fluorescent or radioactive label. With the above ratios of dNTP:dUTP, an average of 1 dU is incorporated into each amplicon. After the PCR reaction, the amplicons are digested with uracil-N-glycosylase and *Escherichia coli* endonuclease IV to remove the uracils and then cut the sugar phosphate backbone of the DNA. Mutations affecting AT base pairs in the test sequence will be revealed by the incorporation of dU and subsequent fragmentation of the amplicon at the site of dU incorporation. The fragments can then be resolved by gel or capillary electrophoresis (Fig. 9-24). Premixed reagents for this assay are available (BESS T-Scan, Epicentre Technologies).

An extension of this method, the BESS G-Tracker, is designed to interrogate G residues in the test sequence.[102] The amplicons, dissolved in a G modification reagent, are subjected to a photoreaction with visible light. A proprietary enzyme mix will then fragment the amplicons at the positions of the modified G residues. Interpretation of the electrophoresis fragment patterns is the same as described above for the T-Scan. BESS is reported to have less optimization requirements than SSCP and ddF.

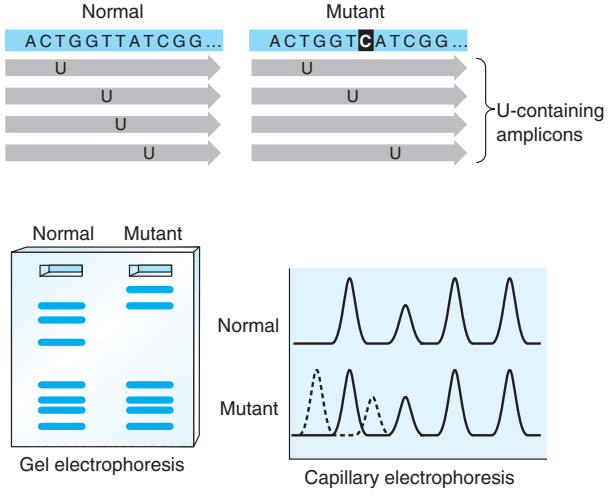


Figure 9-24 Base excision sequence scanning. Uracil containing amplicons yield different digestion fragments, depending on the sequence of the template. Gel (left) or capillary (right) electrophoresis patterns are depicted.

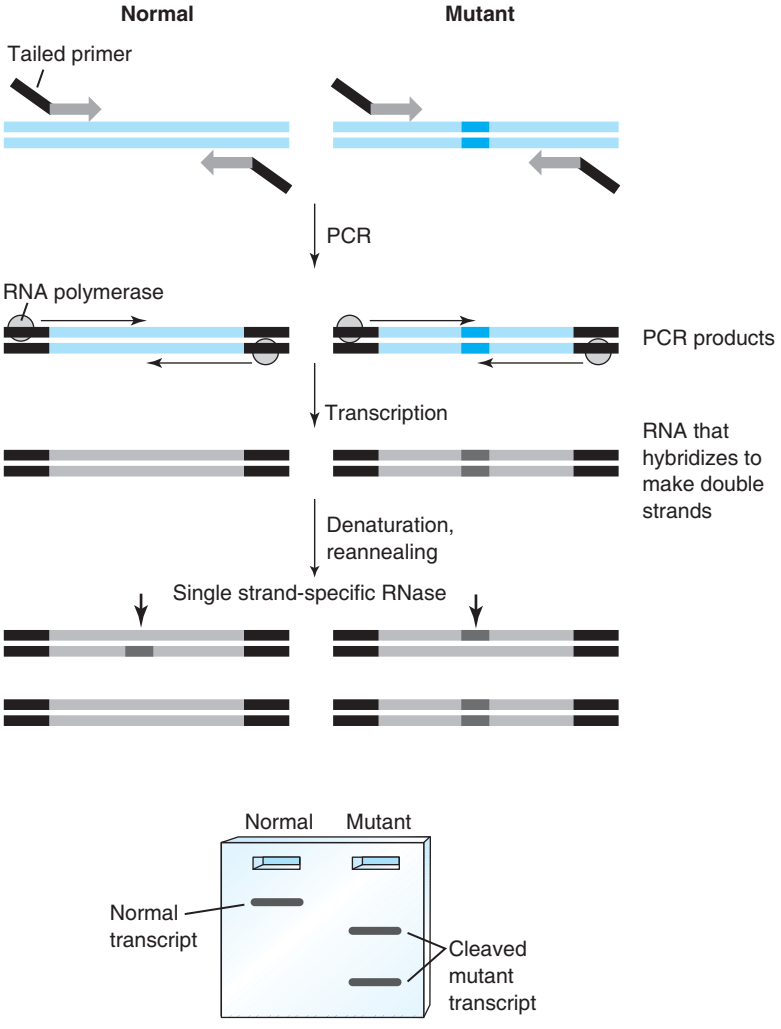
Even so, the complex optimization and interpretation required for BESS preclude its wide use as a clinical test method.

Nonisotopic RNase Cleavage Assay

Nonisotopic RNase cleavage assay (**NIRCA**) is a heteroduplex analysis using duplex RNA.[103] The sequences to be scanned are amplified using primers tailed with promoter sequences of 20–25 bp. T7 or SP6 phage RNA polymerase promoters are most often used for this purpose. Following amplification, the PCR products with the promoter sequences are used as templates for in vitro synthesis of RNA with the T7 or SP6 RNA polymerase enzymes. This reaction yields a large amount of double-stranded RNA (Fig. 9-25). The transcripts are denatured at 95°C and then renatured by cooling to room temperature. If a mutation is present, heteroduplexes form between normal and mutant transcripts. These mismatches in the RNA are targets for cleavage by RNase enzymes. A mixture of single-strand–specific *E. coli* RNase I and *Aspergillus* RNase T1 cleaves different types of mismatches. The remaining double-stranded RNA fragments can then be separated by agarose gel electrophoresis. As in DNA heteroduplex analysis, the size of the RNA fragments implies the placement of the mutation. Although NIRCA has been applied to screening of several clinical targets, including factor IX,[103] p53,[104] and BRCA1,[105,106] it is not widely used in routine patient testing.

Invader Assay

Invader is a method developed by Third Wave Technologies that does not require PCR amplification of samples.[107–109] Premixed reagents are added to a standard 96 well plate along with the test specimens and controls. Included in the reaction mix is the proprietary enzyme Cleavase, which recognizes the structure formed by hybridization of the normal or mutant probes in the mix to the test sequences. During an isothermal incubation, if the probe and test sequence are complementary, two enzymatic cleavage reactions occur, ultimately resulting in a fluorescent signal (Fig. 9-26). The signal can be read by a standard fluorometer. The advantages of this method are the short hands-on time and optional PCR amplification. This method has been applied to several areas of clinical molecular diagnostics, including genetics,[109] hemostasis,[110–112] and infectious disease.[113]



■ Figure 9-25 NIRCA analysis. Normal (left) and mutant (right) transcription templates covering the area to be screened are produced by PCR with tailed primers carrying promoter sequences. RNA polymerase then transcribes the PCR products. The transcripts are denatured and reannealed, forming heteroduplexes between normal and mutant transcripts. RNase cleavage products can be resolved on native agarose gels.

Chemical Cleavage

Chemical cleavage of mismatches (**CCM**) exploits the susceptibility of specific base mismatches to modification by different chemicals.[114] Mismatched cytosines and thymines are modified by hydroxylamine and osmium tetroxide, respectively. For CCM, a labeled normal probe is hybridized to the test sequence; the resulting duplex is treated chemically to modify the bases. Subsequent exposure to a strong reducing agent, piperidine, separates the sugar phosphate backbone of the DNA at the site of the modified bases. The fragments are resolved by polyacrylamide gel electrophoresis. CCM detects only A:A, C:C, G:G, T:T, A:C, A:G, C:T, and G:T mismatches. The ability to detect mutations is extended by using both the sense and antisense strands of the probe. CCM, although highly sensitive, is not attractive for routine analysis due to the hazardous chemicals required and laborious procedure. Still, it is used in some research applications,[115,116] and there have been efforts to automate the process.[117]

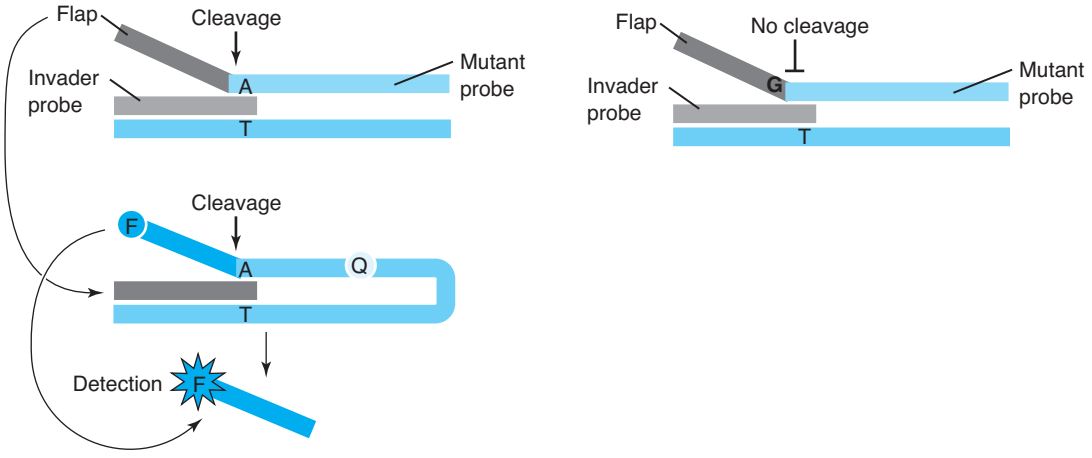


Figure 9-26 Invader single-color assay. Hybridization of supplied probe and anchor sequences to the input template (upper left) forms a structure that is the substrate for the cleavage enzyme. The enzyme removes the flap sequences, which form another hybridization structure with the labeled probe. The second cleavage releases the fluorescent dye from the vicinity of the quencher on the probe, a fluorescent signal. If the template does not match the probe in the first hybridization (upper right), no cleavage occurs.

DNA endonucleases, T4E7 and T7E1 from bacteriophages T4 and T7, also cleave mismatches in DNA.[118] The plant endonuclease CEL 1, with properties similar to the single-stranded nuclease S1, has also been described.[1] Although this method has a higher background than chemical cleavage, it has greater potential for automation and routine use. Commercial kits for this procedure are available (Amersham-Pharmacia). Another commercial enzyme, Surveyor (Transgenomics), is a member of the CEL nuclease family. It cuts both strands of DNA at a mismatch site without regard to the bases involved in the mismatch. This system has been proposed as a screening method for single-base alterations.[119]

Other Methods

The challenges of clinical laboratory requirements for robust, accurate, and sensitive assays have driven the discovery of new techniques and modification of existing techniques.[74,120–122] As a consequence, many methods have been devised, especially for high throughput screening. SSCP is probably the most commonly used mutation screening method in clinical laboratories, but what has been learned from the use of this method is that a single procedure may not be ideal for all genes. Hence the development of DGGE, TTGE, and DHPLC. Combinations of methods have also been proposed to increase sensitivity and detection, such as RFLP and SSCP. The method used in a given laboratory will depend on available instrumentation, the genetic target, and the nature of the mutation.

A summary of methods is shown in Table 9.2. Performance of each method varies, depending on the specimen, template sequence, and type of mutation to be detected. For instance, T-BESS or some chemical cleavage methods that detect only mutations involving specific nucleotides can have 100% accuracy and specificity for these mutations but 0% for mutations affecting other nucleotides. Procedures that are developed by targeting a specific mutation will perform for that target but may not work as well for other targets. For instance, hybridization methods generally detect mutations in GC-rich sequence environments more accurately than in AT-rich sequences. Methods designed to detect defined targets have the best accuracy and specificity; however, they detect only the targeted mutation. Screening methods are required for discovery of new mutations, but these mutations have to be confirmed by other methods or direct sequencing.

Gene Mutation Nomenclature

Accurate testing and reporting of gene mutations require a descriptive and consistent system of expressing mutations and polymorphisms. Recommendations have been reported and generally accepted.[123,124] Following are general descriptive terms for basic alterations and structures.

For DNA and cDNA, the first nucleotide of the first amino acid in the sequence, usually A of ATG for methionine, is designated as position +1. The preceding nucleotide is position +1. There is no nucleotide position 0. Nucleotide changes are expressed as the position or nucleotide interval, the type of nucleotide change, the changed nucleotide, the symbol >, and finally the new nucleotide. For example, consider a nucleotide reference sequence: ATGCGTCACTTA. A substitution of a T for a C at position 7 in the DNA sequence (mutant sequence ATGCGTTACTTA) is expressed as 7C>T. A deletion of nucleotides 6 and 7, ATGCG ACTTA, is expressed as 6_7del or 6_7delTC. An insertion of a TA between nucleotides 5 and 6, ATGCGTATCACTTA, is denoted 5_6insTA.

Duplications are a special type of insertion. A duplication of nucleotides 5 and 6, ATGCGCGTCACTTA, is expressed as 5_6dupCG. An insertion with a concomitant deletion, **indel**, has three alternate descriptive terms. For example, if TC at positions 6 and 7 is deleted from the reference sequence and GACA is inserted, the altered sequence, ATGCGGACAACTTA, is denoted 6_7delTCins GACA, 6_7delinsGACA, or 6_7>GACA. Inversion of nucleotides is designated by the nucleotides affected, inv, and the number of nucleotides inverted. For example, inversion of GCGTCAC starting at position 3 to position 9 in the reference sequence (ATCACTGCGTTA) is 3_9inv7.

Gene mutations in recessive diseases, where both alleles are affected, are indicated by the designation of each mutation separated by +. Thus, a 2357C>T mutation in one allele of a gene and a 2378delA mutation in the other allele on the homologous chromosome is written [2357C>T] + [2378CdelA]. This is distinct from two mutations in the same allele, which is written [2357C>T; 2378CdelA].

Mutations in introns of genomic DNA are indicated by IVS, the intron number, the position of the mutation, and the change. The numbered positions in introns are positive numbers, starting with the G of the GT splice donor site as +1, or negative numbers, starting with the G of the AG splice acceptor site as −1. Thus, a G>T mutation 5 nucleotides from the splice donor site of intron 2 is designated IVS2+5G>T.

At the protein level, numbering begins with the initial amino acid, methionine, in the protein sequence designated +1. The single-letter code has been used to convey protein sequence, but because of concerns about confusion with the single-letter designations, three-letter denotations are also acceptable (see Chapter 3, Table 3.1). Stop codons are designated by X in either case. Amino acid changes are described by the amino acid changed, the position, and the new amino acid. Consider the protein sequence: MRHL. If the second amino acid, arginine (R), was substituted by tyrosine (Y), the mutation of the new amino acid sequence, MYHL, would be R2Y. A nonsense mutation in codon 3, mutant sequence MRX, would be written H3X. Deletion of the arginine and histidine, ML, would be R2_H3del or R2_H3del2. Insertions are denoted by the amino acid interval, ins, and the inserted amino acids. For instance, insertion of amino acids glycine (G), alanine (A), and threonine (T), making the altered amino acid sequence MRGATHL, is indicated by R2_H3insGAT or, alternatively, R2_H3ins3. A short notation for frameshift mutations is the amino acid, its position, and fs. A frameshift mutation affecting the histidine residue changing the amino acid sequence to MRCPLRGWX is simply H3fs. The length of the shifted open reading frame is indicated by adding X and the position of the termination codon. H3CfsX7 is a frameshift in codon 3 that changes a histidine to a cysteine and new reading frame ending in a stop at the seventh codon.

To distinguish between mutation nomenclature referring to genomic DNA, coding (complementary or copy) DNA, mitochondrial DNA, RNA, or protein sequences, a prefix of g., c., m., r., and p. are recommended, respectively. Furthermore, RNA sequences are written in lower case letters. For example, c.89T>C in the coding DNA would be r.89u>c in RNA.

Complex changes and multiple concurrent mutations are reported as they occur. Some mutations, even with sequence information, cannot be positively determined and must be inferred; for example, additions or deletions of repeat units in repeated sequences. For these changes, it is assumed that the 3′ most repeat is the one affected,

and the alteration is noted for that position. Updates and further clarifications of mutation nomenclature are still being addressed. Current information and descriptors for more complex changes are available at genomic.unimelb.edu.au/mdi/mutnomen/

• STUDY QUESTIONS •

1. Name three assays by which the factor V Leiden R506Q mutation can be detected.

2. Exon 4 of the HFE gene from a patient suspected to have hereditary hemachromatosis was amplified by PCR. The G to A mutation, frequently found in hemachromatosis, creates an *Rsa*1 site in exon 4. When the PCR products are digested with *Rsa*1, what results (how many bands) would you expect to see if the patient has the mutation?

3. Which of the following methods would be practical to use to screen a large gene for mutations?
   a. SSP-PCR
   b. SSCP
   c. PCR-RFLP
   d. DGGE
   e. FP-TDI

4. What is the phenotypic consequence of changing a codon sequence from TCT to TCC?

5. A reference sequence, ATGCCCTCTGGC, is mutated in malignant cells. The following mutations in this sequence have been described:
   ATGCGCTCTGGC
   ATGCCCTCGC
   ATAGCCCTCTGGC
   ATGTCTCCCGGC
   ATGCCCTCTGGC
   Express these mutations using the accepted nomenclature.

6. A reference peptide, MPSGCWR, is subject to inherited alterations. The following peptide sequences have been reported:
   MPSTGCWR
   MPSGX
   MPSGCWLVTGX
   MPSGR
   MPSGCWGCWR
   Express these mutations using the accepted nomenclature.

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Chapter 10

Lela Buckingham

DNA Sequencing

OUTLINE

OBJECTIVES

- Compare and contrast the chemical (Maxam/Gilbert) and the chain termination (Sanger) sequencing methods.
- List the components and the molecular reactions that occur in chain termination sequencing.
- Discuss the advantages of dye primer and dye terminator sequencing.
- Derive a text DNA sequence from raw sequencing data.
- Describe examples of alternative sequencing methods, such as bisulfite sequencing and pyrosequencing.
- Define bioinformatics and describe electronic systems for the communication and application of sequence information.
- Recount the events of the Human Genome Project.

In the clinical laboratory, DNA sequence information (the order of nucleotides in the DNA molecule) is used routinely for a variety of purposes, including detecting mutations, typing microorganisms, identifying human haplotypes, and designating polymorphisms. Ultimately, targeted therapies will be directed at abnormal DNA sequences detected by these techniques.[1]

Direct Sequencing

The importance of knowing the order, or sequence, of nucleotides on the DNA chain was appreciated in the earliest days of molecular analysis. Elegant genetic experiments with microorganisms detected molecular changes indirectly at the nucleotide level.

Indirect methods of investigating nucleotide sequence differences are still in use. Molecular techniques, from Southern blot to the mutation detection methods described in Chapter 9, are aimed at identifying nucleotide changes. Without knowing the nucleotide sequence of the targeted areas, results from many of these methods would be difficult to interpret; in fact, some methods would not be useful at all. Direct determination of the nucleotide sequence, or DNA **sequencing**, is the most definitive molecular method to identify genetic lesions.

Manual Sequencing

Direct determination of the order, or sequence, of nucleotides in a DNA polymer is the most specific and direct method for identifying genetic lesions (mutations) or polymorphisms, especially when looking for changes affecting only one or two nucleotides. Two types of sequencing methods have been used most extensively: the **Maxam-Gilbert method**[2] and the **Sanger method.**[3]

Chemical (Maxam-Gilbert) Sequencing

The Maxam-Gilbert chemical sequencing method was developed in the late 1970s by Allan M. Maxam and Walter Gilbert. Maxam-Gilbert sequencing requires a double- or single-stranded version of the DNA region to be sequenced, with one end radioactively labeled.

For sequencing, the labeled fragment, or **template,** is aliquoted into four tubes. Each aliquot is treated with a different chemical with or without high salt (Fig. 10-1). Upon addition of a strong reducing agent, such as 10% piperidine, the single-stranded DNA will break at specific nucleotides (Table 10.1).

After the reactions, the piperidine is evaporated, and the contents of each tube are dried and resuspended in formamide for gel loading. The fragments are then separated by size on a denaturing polyacrylamide gel (Chapter 5). The denaturing conditions (formamide, urea, and heat) prevent the single strands of DNA from hydrogen bonding with one another or folding up so that they

Advanced Concepts

To make a radioactive sequence template, (γ-^{32}P) ATP can be added to the 5′ end of a fragment, using T4 polynucleotide kinase, or the 3′ end, using terminal transferase plus alkaline hydrolysis to remove excess adenylic acid residues. Double-stranded fragments labeled only at one end are also produced by using restriction enzymes to cleave a labeled fragment asymmetrically, and the cleaved products are isolated by gel electrophoresis. Alternatively, denatured single strands are labeled separately, or a "sticky" end of a restriction site is filled in incorporating radioactive nucleotides with DNA polymerase.

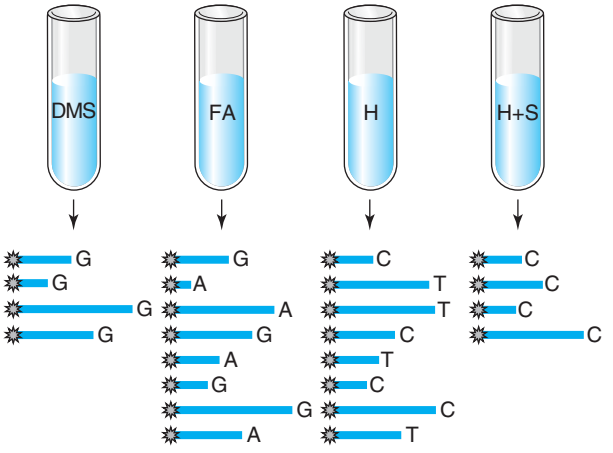


Figure 10-1 Chemical sequencing proceeds in four separate reactions in which the labeled DNA fragment is selectively broken at specific nucleotides. (DMS-dimethylsulphate; FA-formic acid; H-hydrazine; H+S=hydrazine+salt)

Table 10.1 **Specific Base Reactions in Maxam-Gilbert Sequencing**

Chain breaks at:	Base Modifier	Reaction	Time (min at 25°C)
G	Dimethylsulphate	Methylates G	4
G + A	Formic acid	Protonates purines	5
T + C	Hydrazine	Splits pyrimidine rings	8
C	Hydrazine + salt	Splits only C rings	8

migrate through the gel strictly according to their size. The migration speed is important because single-base resolution is required to interpret the sequence properly.

After electrophoresis, the gel apparatus is disassembled; the gel is removed to a sheet of filter paper, and it is dried on a gel dryer. The dried gel is exposed to light-sensitive film. Alternatively, wet gels can be exposed directly. An example of Maxam-Gilbert sequencing results is shown in Figure 10-2. The sequence is inferred from the bands on the film. The smallest (fastest-migrating) band represents the base closest to the labeled end of the fragment. The lane in which that band appears identifies the nucleotide. Bands in the purine (G + A) or pyrimidine (C + T) lane are called based on whether they are also present in the G- or C-only lanes. Note how the sequence is read from the bottom (5′ end of the DNA molecule) to the top (3′ end of the molecule) of the gel.

Although Maxam-Gilbert sequencing is a relatively efficient way to determine short runs of sequence data, the method is not practical for high throughput sequencing of long fragments. In addition, the hazardous chemicals hydrazine and piperidine require more elaborate precautions for use and storage. This method has therefore been replaced by the dideoxy chain termination sequencing method for most sequencing applications.

Advanced Concepts

Polyacrylamide gels from 6% to 20% are used for sequencing. Bromophenol blue and xylene cyanol loading dyes are used to monitor the migration of the fragments. Run times range 1–2 hours for short fragments (up to 50 bp) to 7–8 hours for longer fragments (more than 150 bp).

Dideoxy (Sanger) Sequencing

The original **dideoxy chain termination** sequencing methods required a single-stranded template. Templates up to a few thousand bases long could be produced using **M13** bacteriophage, a bacterial virus with a single-stranded DNA genome. This virus replicates by infecting *Escherichia coli*, in which the viral single-stranded circular genome is converted to a double-stranded plasmid, called the replication factor (RF). The plasmid codes for

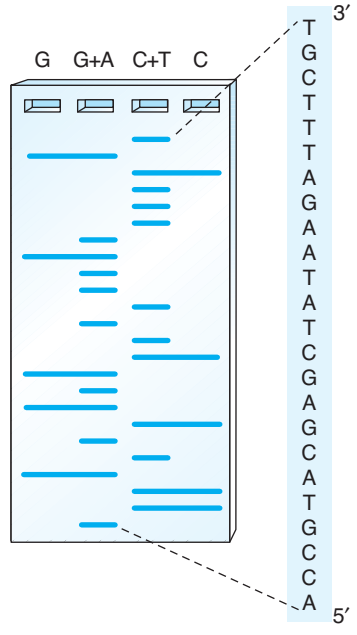


Figure 10-2 Products of a Maxam-Gilbert sequencing reaction. The gel is read from the bottom to the top. The size of the fragments gives the order of the nucleotides. The nucleotides are inferred from the lane in which each band appears. A or T is indicated by bands that appear in the G + A lane or C + T lane, respectively, but not in the G lane or the C lane. G is present in the G + A lane and the G lane. C is present in the C + T lane and the C lane.

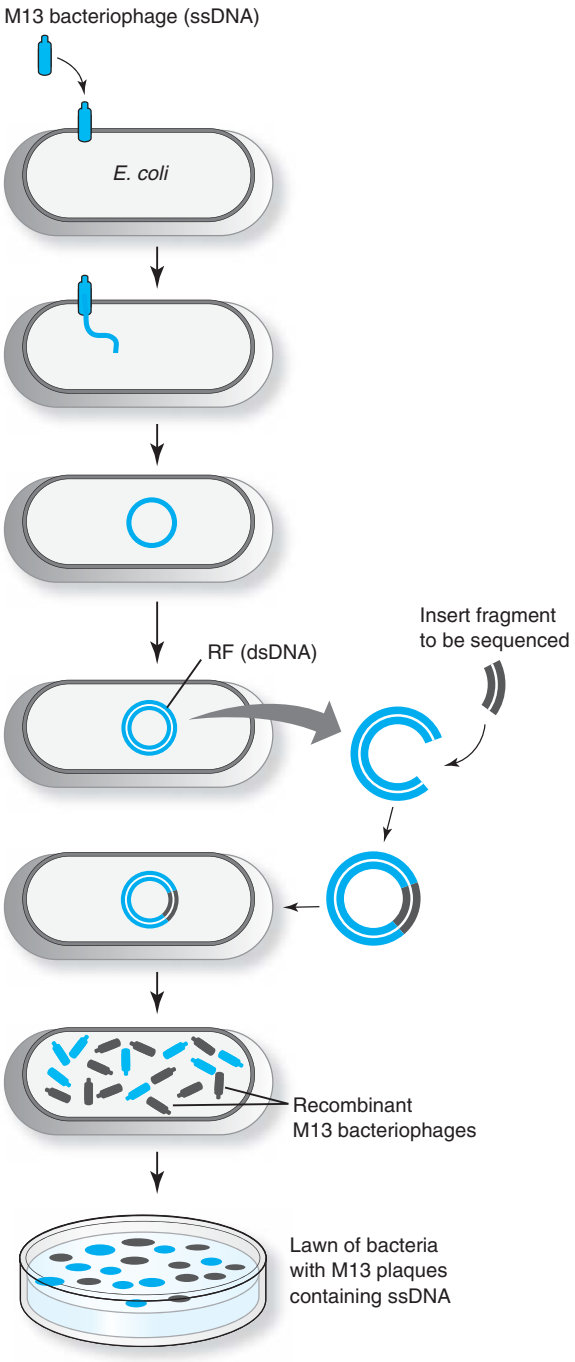
viral gene products that use the bacterial transcription and translation machinery to make new single-stranded genomes and viral proteins. To use M13 for template preparation, the RF is isolated from infected bacteria, cut with restriction enzymes, and the fragment to be sequenced is ligated into the RF (Fig. 10-3). When the recombined RF is reintroduced into the host bacteria, M13 continues its life cycle producing new phages, some of which carry the inserted fragment. When the phages are spread on a lawn of host bacteria, plaques (clear spaces) of lysed bacteria formed by phage replication contain pure populations of recombinant phage. The single-stranded DNA can then be isolated from the phage by picking plugs of agar from the plaques and boiling them to isolate the single-stranded phage DNA.

Dideoxy chain termination (Sanger) sequencing is a modification of the DNA replication process. A short, synthetic single-stranded DNA fragment (primer) complementary to sequences just 5′ to the region of DNA to be sequenced is used for priming dideoxy sequencing reactions (Fig. 10-4). For detection of the products of the sequencing reaction, the primer may be attached covalently at the 5′ end to a ^{32}P-labeled nucleotide or a fluorescent dye-labeled nucleotide. An alternative detection strategy is to incorporate ^{32}P- or ^{35}S-labeled deoxynucleotides in the nucleotide sequencing reaction mix. The latter is called **internal labeling**.

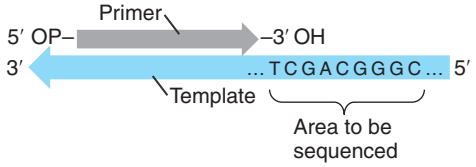
Just as in the in vivo DNA replication reaction, an in vitro DNA synthesis reaction would result in polymerization of deoxynucleotides to make full-length copies of the DNA template (DNA replication is discussed in Chapter 1). For sequencing, modified **dideoxynucleotide** (ddNTP) derivatives are added to the reaction mixture. Dideoxynucleotides lack the hydroxyl group found on the 3′ ribose carbon of the deoxynucleotides (dNTPs;

Advanced Concepts

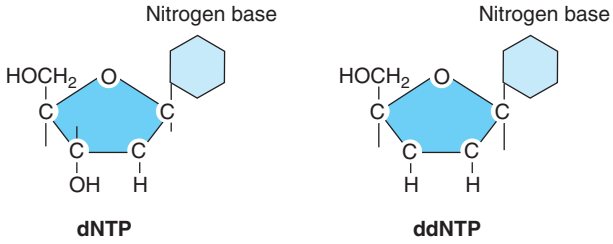
Because of extensive use of M13, a primer that hybridizes to M13 sequences could be used to sequence any fragment. This primer, the **M13 universal primer**, is still used in some applications, even though the M13 method of template preparation is no longer practical.



■ Figure 10-3 Preparation of single-stranded sequencing template using M13 bacteriophage. The engineered RF is replicated as the phage genome. The template is isolated from plaques made by pure clones of recombinant phage on a lawn of bacteria.



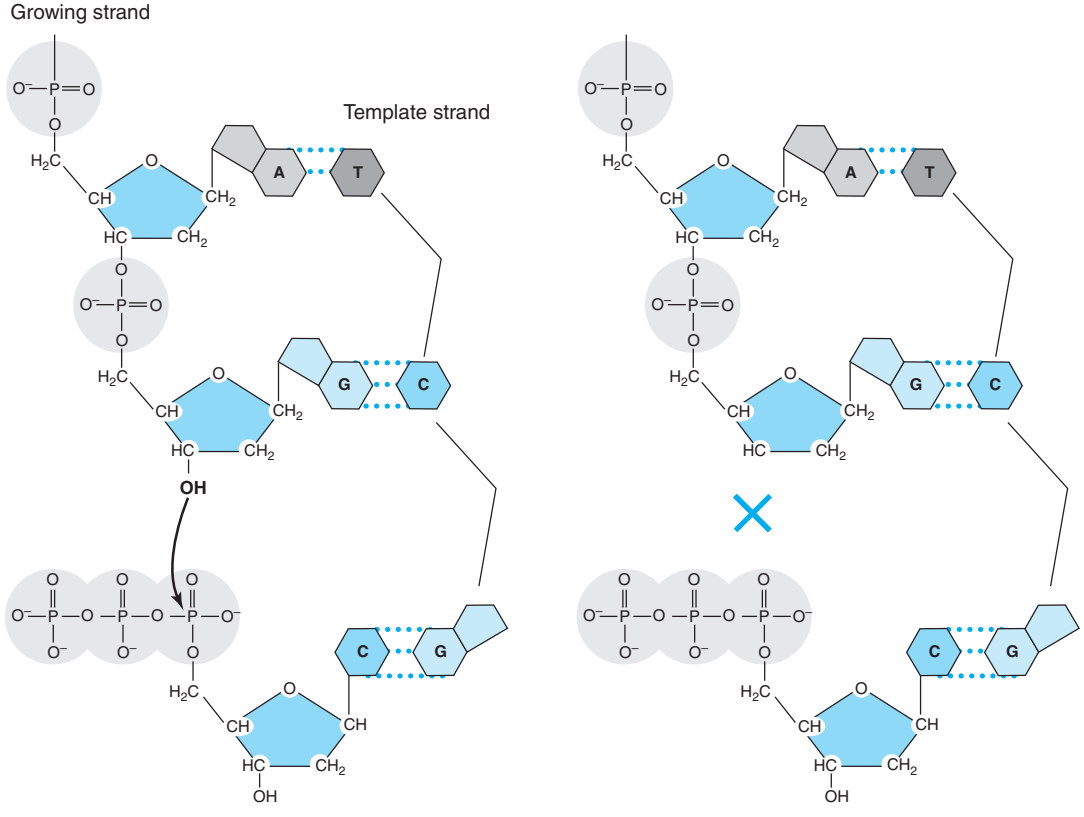
■ Figure 10-4 Manual dideoxy sequencing requires a single-stranded version of the fragment to be sequenced (template). Sequencing is primed with a short synthetic piece of DNA complementary to bases just before the region to be sequenced (primer). The sequence of the template will be determined by extension of the primer in the presence of dideoxynucleotides.



■ Figure 10-5 A dideoxynucleotide (right) lacks the hydroxyl group on the 3′ ribose carbon that is required for formation of a phosphodiester bond with the phosphate group of another nucleotide.

Fig. 10-5). DNA synthesis will stop upon incorporation of a ddNTP into the growing DNA chain (chain termination) because without the hydroxyl group at the 3′ sugar carbon, the 5′-3′ phosphodiester bond cannot be established to incorporate a subsequent nucleotide. The newly synthesized chain will terminate, therefore, with the ddNTP (Fig. 10-6).

To perform a manual dideoxy sequencing reaction, a 1:1 mixture of template and primer is placed into four separate reaction tubes in sequencing buffer (Fig. 10-7).



■ Figure 10-6 DNA replication (left) is terminated by the absence of the 3′ hydroxyl group on the dideoxyguanosine nucleotide (ddG, right). The resulting fragment ends in ddG.

Advanced Concepts

PCR products are often used as sequencing templates. It is important that the amplicons to be used as sequencing templates are free of residual components of the PCR reaction, especially primers and nucleotides. These reactants can interfere with the sequencing reaction and lower the quality of the sequencing ladder. PCR amplicons can be cleaned by adherence and washing on solid phase (column or bead) matrices, alcohol precipitation, or enzymatic digestion with alkaline phosphatase. Alternatively, amplicons can be run on an agarose gel and the bands eluted. The latter method provides not only a clean template but also confirmation of the product being sequenced. It is especially useful when the PCR reactions are not completely free of misprimed bands or primer dimers (see Chapter 7).

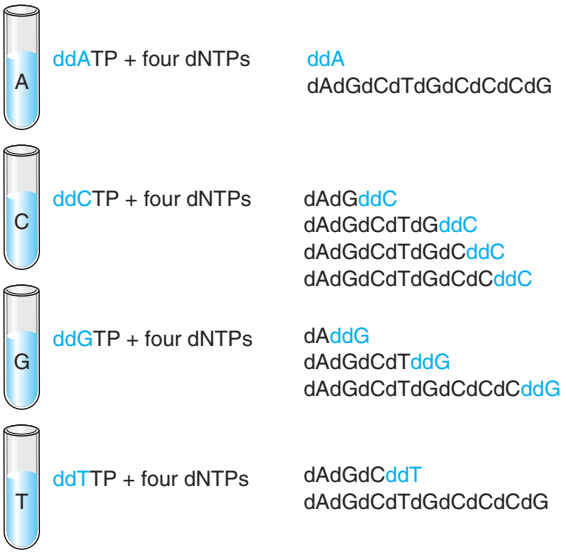


Figure 10-7 Components required for DNA synthesis (template, primer, enzyme, buffers, dNTPs) are mixed with a different ddNTP in each of four tubes (left). With the proper ratio of ddNTPs:dNTPs, the newly synthesized strands of DNA will terminate at each opportunity to incorporate a ddNTP. The resulting synthesis products are a series of fragments ending in either A (ddATP), C (ddCTP), G (ddGTP) or T (ddTTP). This collection of fragments is the sequencing ladder.

Sequencing buffer is usually provided with the sequencing enzyme and contains ingredients necessary for the polymerase activity. Mixtures of all four dNTPs and one of the four ddNTPs are then added to each tube, with a different ddNTP in each of the four tubes.

The ratio of ddNTPs:dNTPs is critical for generation of a readable sequence. If the concentration of ddNTPs is too high, polymerization will terminate too frequently early along the template. If the ddNTP concentration is too low, infrequent or no termination will occur. In the beginning days of sequencing, optimal ddNTP:dNTP ratios were determined empirically (by experimenting with various ratios). Modern sequencing reagent mixes have preoptimized nucleotide mixes.

With the addition of DNA polymerase enzyme to the four tubes, the reaction begins. After about 20 minutes, the reactions are terminated by addition of a stop buffer. The stop buffer consists of 20 mM EDTA to chelate cations and stop enzyme activity, formamide to denature the products of the synthesis reaction, and gel loading dyes (bromophenol blue and/or xylene cyanol). It is important that all four reactions be carried out for equal time. Maintaining equal reaction times will provide consistent band intensities in all four lanes of the gel sequence, which facilitates final reading of the sequence.

Advanced Concepts

Manganese (Mn^{++}) may be added to the sequencing reaction to promote equal incorporation of all dNTPs by the polymerase enzyme.[31,32] Equal incorporation of the dNTPs makes for uniform band intensities on the sequencing gel, which eases interpretation of the sequence. Manganese increases the relative incorporation of ddNTPs as well, which will enhance the reading of the first part of the sequence by increasing intensity of the smaller bands on the gel. Modified nucleotides, deaza-dGTP and deoxyinosine triphosphate (dITP), are also added to sequencing reaction mixes to deter secondary structure in the synthesized fragments. Additives such as Mn^{++}, deaza-dGTP, and dITP are supplied in preoptimized concentrations in commercial sequencing buffers.

The sets of synthesized fragments are then loaded onto a denaturing polyacrylamide gel (see Chapter 5 for more details about polyacrylamide gel electrophoresis). The products of each of the sequencing reactions are loaded into four adjacent lanes, labeled A, C, G, or T, corresponding to the ddNTP in the four reaction tubes. Once the gel is dried and exposed to x-ray film, the fragment patterns can be visualized from the signal on the ^{32}P-labeled primer or nucleotide. All fragments from a given tube will end in the same ddNTP; for example, all the fragments synthesized in the ddCTP tube end in C. The four-lane gel electrophoresis pattern of the products of the four sequencing reactions is called a **sequencing ladder** (Fig. 10-8). The ladder is read to deduce the DNA sequence. From the bottom of the gel, the smallest (fastest-migrating) fragment is the one in which synthesis terminated closest to the primer. The identity of the ddNTP at a particular position is determined by the lane in which the band appears. If the smallest band is in the ddATP lane, then the first base is an A. The next larger fragment is the one that was terminated at the next position on the template. The lane that has the next larger band identifies the next nucleotide in the sequence. In the figure, the next largest band is found in the ddGTP lane, so the next base is a G. The sequence is thus read from the bottom (smallest, 5′-most) to the top (largest, 3′-most) fragments across or within lanes to determine the identity and order of nucleotides in the sequence.

Depending on the reagents and gel used, the number of bases per sequence read averages 300–400. Advances in enzyme and gel technology have increased this capability to over 500 bases per read. Sequencing reads can also be lengthened by loading the same ladders in intervals of 2–6 hours so that the larger bands are resolved with longer (e.g., 8-hour) migrations, whereas smaller bands will be resolved simultaneously in a 1–2–hour migration that was loaded 6–7 hours later.

Sequencing technology has been improved significantly from the first routine manual sequencing procedures. Recombinant polymerase enzymes, such as Sequenase,[4] and the heat stable enzymes Thermosequenase[5] and Therminator are now available; in vitro removal of the exonuclease activity of these enzymes makes them faster and more processive (i.e., they stay with the tem-

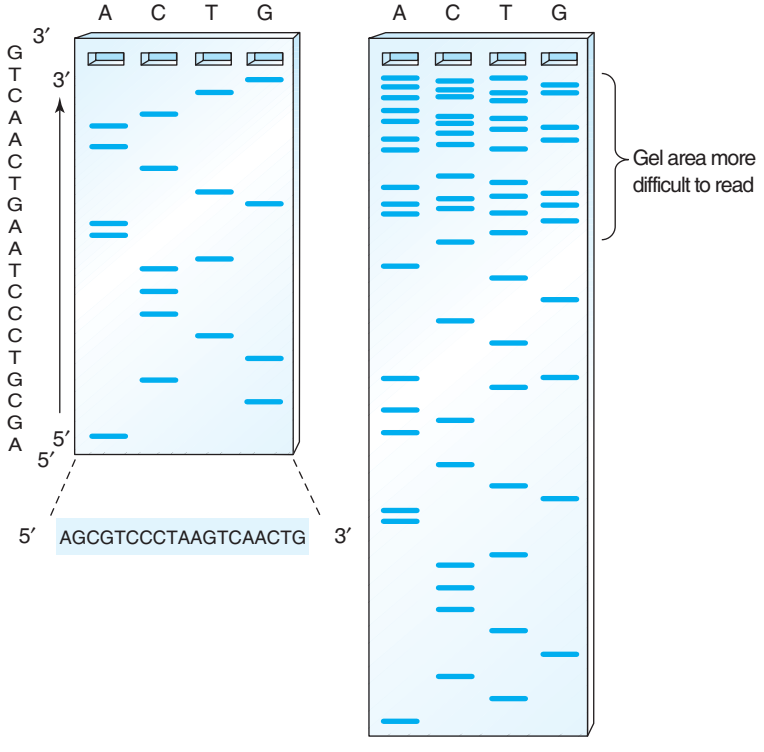


Figure 10-8 A sequencing ladder is read from the bottom of the gel to the top. The smallest (fastest migrating) fragment represents the first nucleotide attached to the primer by the polymerase. Since that fragment is in lane A, from the reaction that contained ddATP (left), the sequence read begins with A. The next largest fragment is in lane G. The sequence, then, reads AG. The next largest fragment is in lane C, making the sequence AGC, and so forth up the gel. Larger bands on a sequencing gel can sometimes be compressed, limiting the length of sequence that can be read on a single gel run (right).

plate longer, producing longer sequencing ladders). In addition, these engineered enzymes more efficiently incorporate ddNTPs and nucleotide analogs such as dITP (deoxyinosine triphosphate) or 7-deaza-dGTP, which are used to deter secondary structure (internal folding and hybridization) in the template and sequencing products. Furthermore, most sequencing methods in current use are performed with double-stranded templates, eliminating the tedious preparation of single-stranded versions of the DNA to be sequenced.

Using the heat-stable enzymes such as Therminator and Thermosequenase, the sequencing reaction can be performed in a thermal cycler (**cycle sequencing**). With cycle sequencing, timed manual starting and stopping of the sequencing reactions are not necessary. The labor savings in this regard increase the number of reactions that can be performed simultaneously; for example, a single operator can set up 96 sequencing reactions (i.e., sequence 24 fragments) in a 96-well plate. Finally, improvements in fluorescent dye technology have led to the automation of the sequencing process and, more importantly, sequence determination.

Automated Fluorescent Sequencing

The chemistry for automated sequencing is the same as described for manual sequencing, using double-stranded templates and cycle sequencing. Because cycle sequencing (unlike manual sequencing) does not require sequential addition of reagents to start and stop the reaction, cycle sequencing is more easily adaptable to high-throughput applications and automation.[6] Universal systems combine automation of DNA isolation of the template and setup of the sequencing reactions. For example, the Qiagen BioRobot 9600 can isolate template DNA and set up the sequencing reactions for cycle sequencing of 48 samples in 35 minutes.

Electrophoresis and reading of the sequencing ladder can also be automated. A requirement for automated reading of the DNA sequence ladder is the use of fluorescent dyes instead of radioactive nucleotides to label the primers or sequencing fragments.

Fluorescent dyes used for sequencing have distinct "colors," or peak wavelengths of fluorescence emission, that can be distinguished by automated sequencers. The advantage of having four distinct colors is that all four of the reaction mixes can be read in the same lane of a gel or on a capillary. Fluorescent dye color rather than lane placement will assign the fragments as ending in A, T, G, or C in the sequencing ladder (Fig. 10-9).

Advanced Concepts

Fluorescent dyes used for automated sequencing include fluorescein and rhodamine dyes and Bodipy (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) dye derivatives that are recognized by commercial detection systems.[16,33] Automated sequence readers excite the dyes with a laser and detect the emitted fluorescence at predetermined wavelengths. More advanced methods have been proposed to enhance the distinction between the dyes for more accurate determination of the sequence.[34]

Approaches to Automated Sequencing

There are two approaches to automated fluorescent sequencing: **dye primer** and **dye terminator** sequencing (Fig. 10-10). The goal of both approaches is the same: to label the fragments synthesized during the sequencing reaction according to their terminal ddNTP. Thus, fragments ending in ddATP, read as A in the sequence, will be labeled with a "green" dye; fragments ending in ddCTP, read as C in the sequence, will be labeled with a "blue"

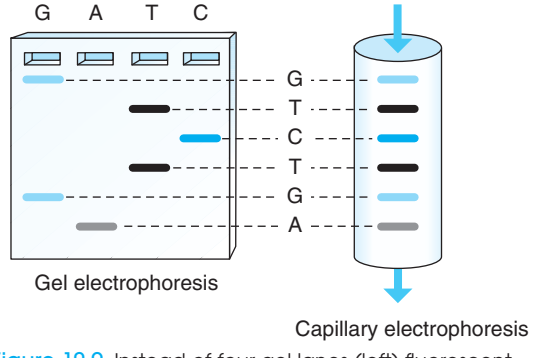
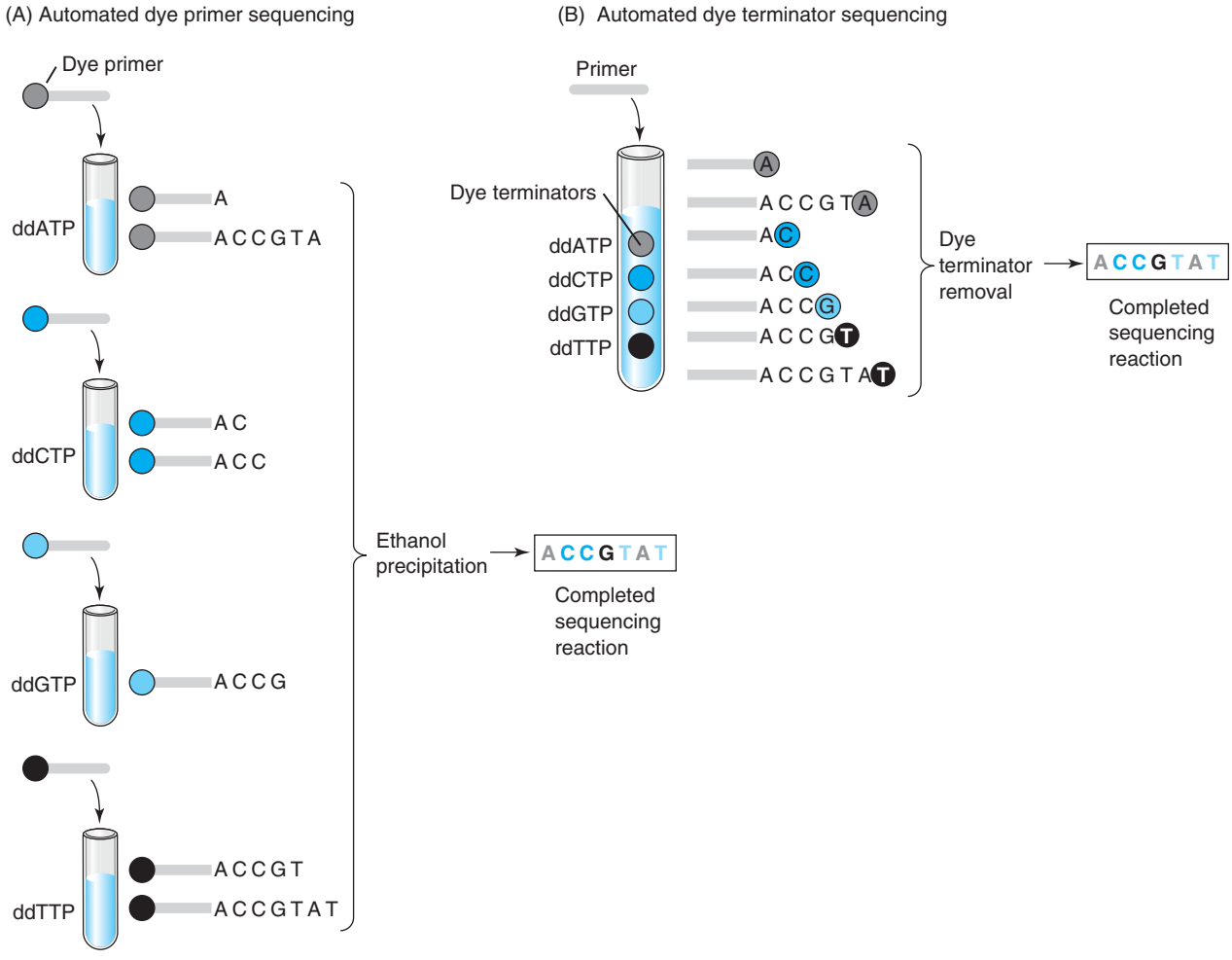


Figure 10-9 Instead of four gel lanes (left) fluorescent fragments can be run in a single gel lane or in a capillary (right). Note that the sequence of nucleotides, AGTCTG, read by lane in the slab gel is read by color in the capillary.



■ Figure 10-10 Fluorescent sequencing chemistries. Dye primer sequencing uses labeled primers (A). The products of all four reactions are resolved together in one lane of a gel or in a capillary. Using dye terminators (B) only one reaction tube is necessary, since the fragments can be distinguished directly by the dideoxynucleotides on their 3′ ends.

dye; fragments ending in ddGTP, read as G in the sequence, will be labeled with a "black" or "yellow" dye; and fragments ending in ddTTP, read as T in the sequence, will be labeled with a "red" dye. This facilitates reading of the sequence by the automated sequence.

In dye primer sequencing, the four different fluorescent dyes are attached to four separate aliquots of the primer. The dye molecules are attached covalently to the 5′ end of the primer during chemical synthesis, resulting in four versions of the same primer with different dye labels. The primer labeled with each "color" is added to four separate reaction tubes, one each with ddATP, ddCTP, ddGTP, or ddTTP, as shown in Figure 10-10. After addition of the rest of the components of the sequencing reaction (see the section above on manual sequencing) and of a heat stable polymerase, the reaction is subjected to cycle sequencing in a thermal cycler. The products of the sequencing reaction are then labeled at the 5′ end, the dye color associated with the ddNTP at the end of the fragment.

Dye terminator sequencing is performed with one of the four fluorescent dyes attached to each of the ddNTPs instead of to the primer. The primer is unlabeled. A major advantage of this approach is that all four sequencing reactions are performed in the same tube (or well of a plate) instead of in four separate tubes. After addition of the rest of the reaction components and cycle sequencing, the product fragments are labeled at the 3′ end. As with dye primer sequencing, the "color" of the dye corresponds to the ddNTP that terminated the strand.

Preparation of the Sequencing Ladder

After a sequencing reaction using fluorescent dye terminators, excess dye terminators must be removed from the sequencing ladder. Sequencing ladders can be cleaned with columns or beads or by ethanol precipitation. Most spin columns or bead systems bind the sequencing fragments to allow removal of residual sequencing components by rinsing with buffers. Another approach is to bind the dye terminators onto specially formulated magnetic beads and recover the DNA ladder from the supernatant as the beads are held by a magnet applied to the outside of the tube or plate.

The fragments of the sequencing ladder should be completely denatured before running on a gel or capillary. Denaturing conditions (50°–60°C, formamide, urea denaturing gel) should be maintained as the fragments must be resolved strictly according to size. Secondary structure can affect migration speed and lower the quality of the sequence. Before loading in a gel or capillary instrument, sequence ladders are cleaned, as described above, to remove residual dye terminators, precipitated, and resuspended in formamide. The ladders are heated to 95°–98°C for 2–5 minutes and placed on ice just before loading.

Electrophoresis and Sequence Interpretation

Both dye primer and dye terminator sequencing reactions are loaded onto a slab gel or capillary gel in a single lane. The fluorescent dye colors, rather than lane assignment, distinguish which nucleotide is at the end of each fragment. Running all four reactions together not only increases throughput but also eliminates lane-to-lane migration variations that affect accurate reading of the sequence. The fragments migrate through the gel according to size and pass in turn by a laser beam and a detector in the automated sequencer. The laser beam excites the dye attached to each fragment, causing the dye to emit fluorescence that is captured by the detector. The detector converts the fluorescence to an electrical signal that is recognized by computer software as a flash or peak of color.

Fluorescent detection equipment yields results as an **electropherogram**, rather than a gel pattern. Just as the gel sequence is read from the smallest (fastest-migrating) fragments to the largest, the automated sequencer reads, or "calls," the bases from the smallest (fastest-migrating) fragments that first pass the detector to the largest. The instrument calls the base by the color of the fluorescence of the fragment as it passes the detector. The readout from the instrument is a series of peaks of the four fluorescent dyes as the bands of the sequencing ladder migrate by the detector. The software assigns one of four arbitrary colors (associated with each of the fluorescent dyes) and a text letter to the peaks for ease of interpretation.

As with manual sequencing, the ratio of ddNTPs: dNTPs is key to the length of the sequence read (how much of the template sequence can be determined). Too many ddNTPs will result in a short sequence read. Too low a concentration of ddNTPs will result in loss of sequence data close to the primer but give a longer read, because the sequencing enzyme will polymerize further down the template before it incorporates a ddNTP into the growing chain. The quality of the sequence (height and separation of the peaks) improves away from the primer and begins to decline at the end. At least 400–500 bases can be easily read with most sequencing chemistries.

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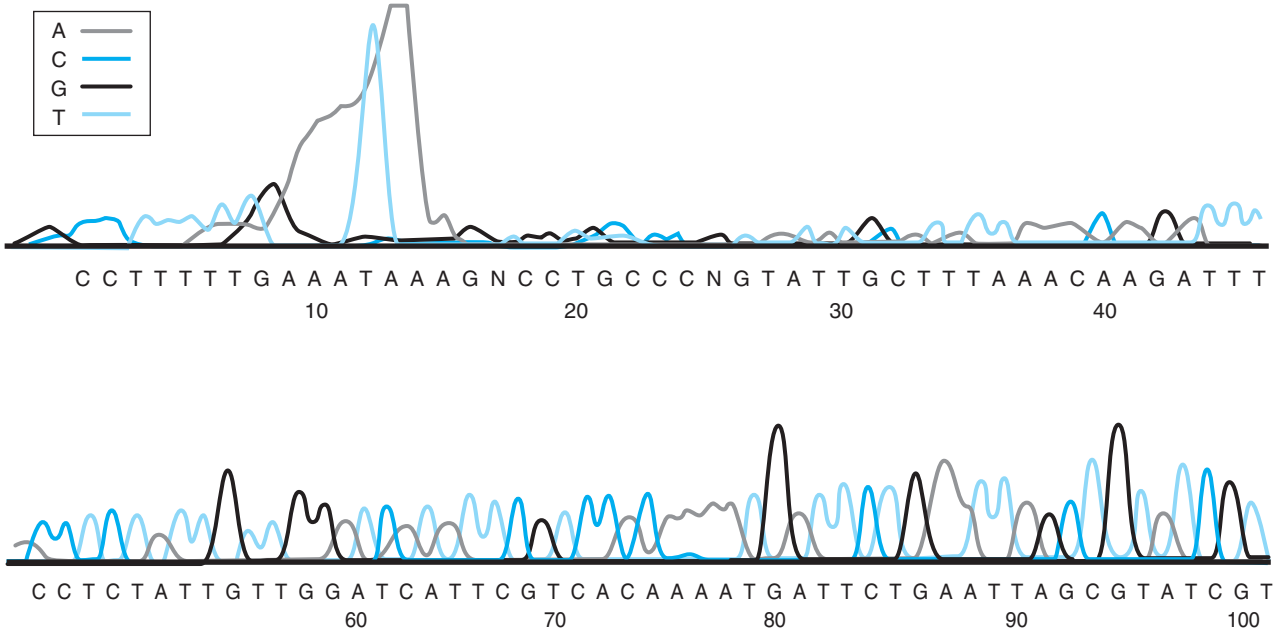
DNA sequences with high G/C content are sometimes difficult to read due to intrastrand hybridization in the template DNA. Reagent preparations that include 7-deaza-dGTP (2′-deoxy-7-deazaguanosine triphosphate) or deoxyinosine triphosphate instead of standard dGTP improve the resolution of bands in regions that exhibit GC **band compressions** or bunching of bands close together so that they are not resolved, followed by several bands running farther apart.



■ Figure 10-11 Electropherogram showing a dye blob at the beginning of a sequence (positions 9-15). The sequence read around this area is not accurate.

Interpretation of sequencing data from a dye primer or dye terminator reaction is not always straightforward. The quality of the electropherogram depends on the quality of template, the efficiency of the sequencing reaction, and the cleanliness of the sequencing ladder. Failure to clean the sequencing ladder properly results in bright flashes of fluorescence (**dye blobs**) that obliterate parts of the sequence read (Fig. 10-11). Poor starting material results in a poor-quality sequence that cannot be read accurately (Fig. 10-12). Clear, clean sequencing ladders are read accurately by the automated reader, and a text sequence is generated. Sequencing software indicates the certainty of each base call in the sequence. Some programs compare two sequences or test with reference sequences to identify mutations or polymorphisms. Less than optimal sequences are not accurately readable by automated detectors but can sometimes be read by an experienced operator.

It is important to sequence both strands of DNA to confirm sequence data. This is critical for confirmation of mutations or polymorphisms in a sequence (Fig. 10-13). Alterations affecting a single base pair can be subtle on an electropherogram, especially if the alteration is in the heterozygous form. Ideally, a heterozygous mutation appears as two peaks of different color directly on top of one another; that is, at the same position in the electropherogram. The overlapping peaks should be about half the height of the rest of the sequence. Heterozygous deletions or insertions (*e.g.*, the *BRCA* frameshift mutations) affect all positions of the sequence downstream of the mutation (Fig. 10-14) and, thus, are more easily detected. Somatic mutations in clinical specimens are sometimes most difficult to detect as they may be diluted by normal sequences that mask the somatic change.

Several software applications have been written to interpret and apply sequence data from automatic sequencers. Software that collects the raw data from the instrument is supplied with automated sequencing instruments. Software that interprets, compares, or otherwise manipulates sequence data is sometimes supplied with a purchased instrument or available on the Internet. A representative sample of these applications is shown in Table 10.2.

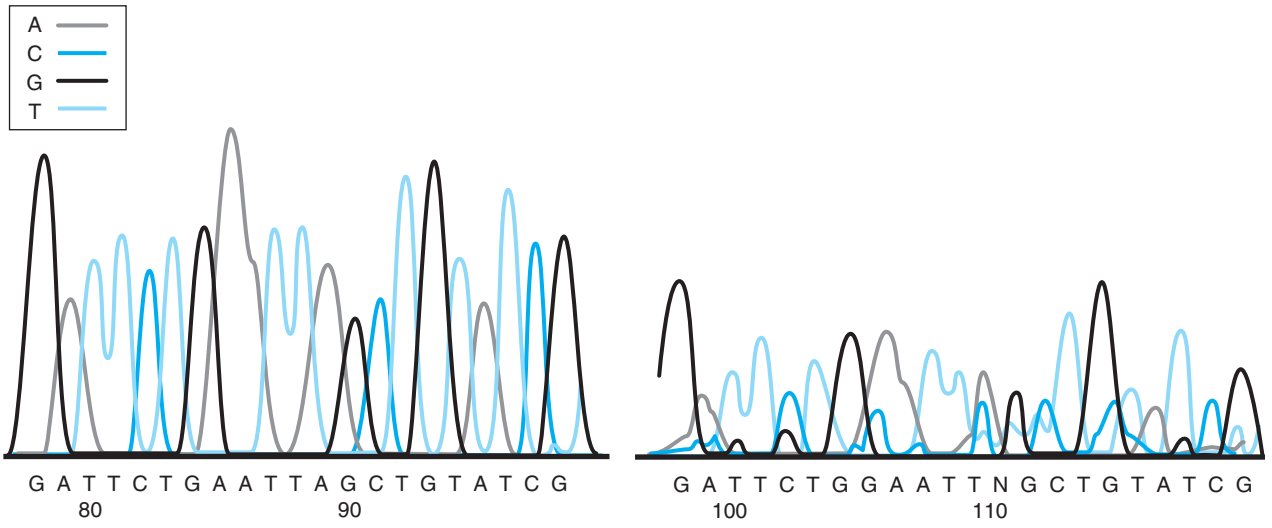


Figure 10-12 Examples of good sequence quality (left) and poor sequence quality (right). Note the clean baseline on the good sequence; that is, only one color peak is present at each nucleotide position. Automatic sequence reading software will not accurately call a poor sequence. Compare the text sequences above the two scans.

Pyrosequencing

Chain termination sequencing is the most widely used method to determine DNA sequence. Other methods have been developed that yield the same information but not with the throughput capacity of the chain termination method. Pyrosequencing is an example of a method designed to determine a DNA sequence without having to make a sequencing ladder.[7,8] This procedure relies on the generation of light (luminescence) when nucleotides are added to a growing strand of DNA (Fig. 10-15). With this system, there are no gels, fluorescent dyes, or ddNTPs.

The pyrosequencing reaction mix consists of a single-stranded DNA template, sequencing primer, sulfurylase and luciferase, plus the two substrates adenosine 5′ phosphosulfate (APS) and luciferin. Sequentially, one of the four dNTPs is added to the reaction. If the nucleotide is complementary to the base in the template strand next to

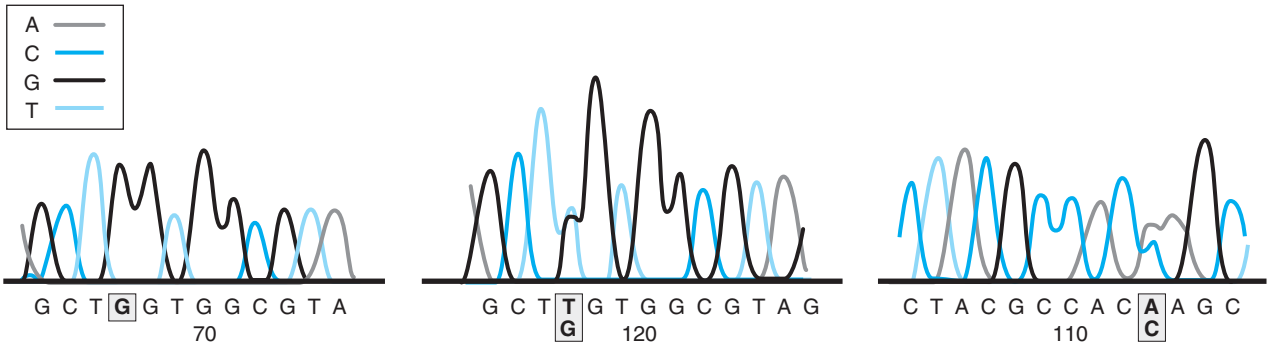


Figure 10-13 Sequencing of a heterozygous G>T mutation in exon 12 of the ras gene. The normal codon sequence is GGT (right). The heterozygous mutation, (G/T) (center) is confirmed in the reverse sequence, (C/A) (right).

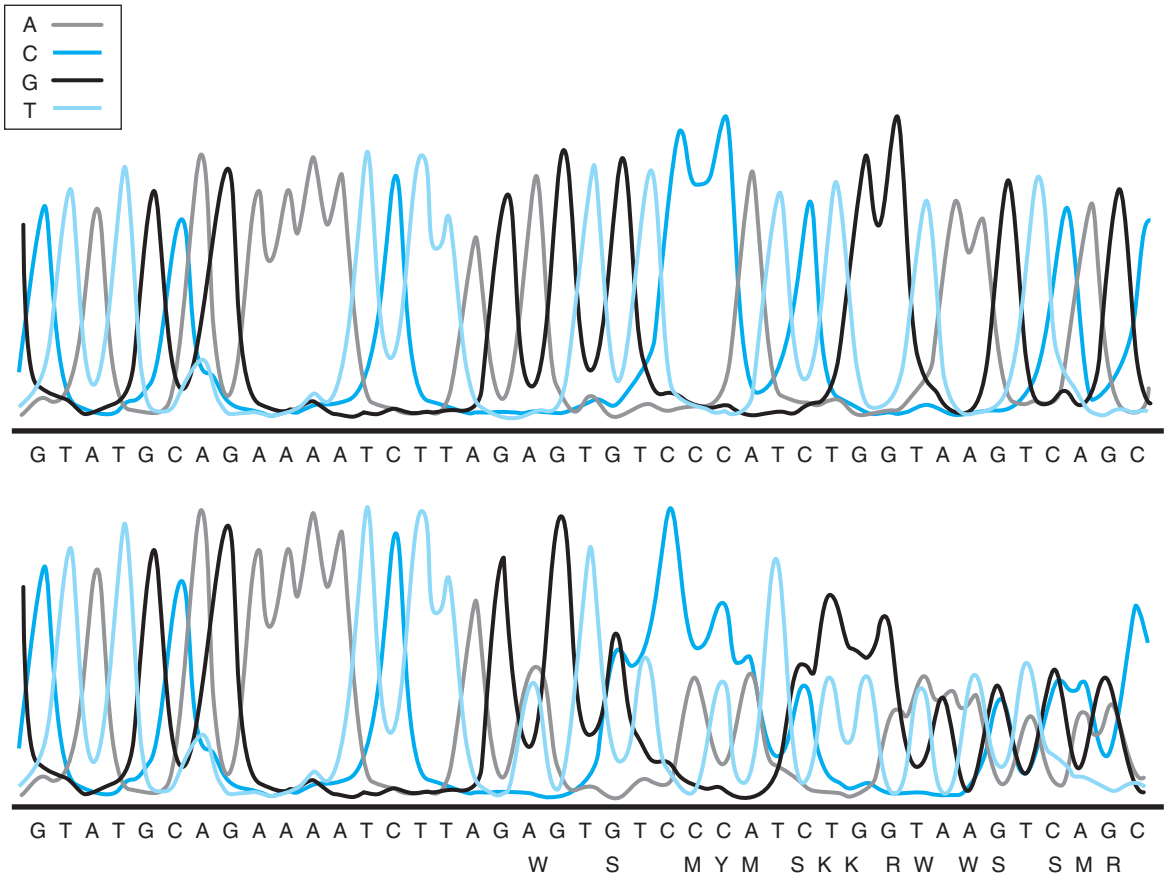


Figure 10-14 187 delAG mutation in the *BRCA*1 gene. This heterozygous dinucleotide deletion is evident in the lower panel where, at the site of the mutation, two sequences are overlaid: the normal sequence and the normal sequence minus two bases.

Table 10.2 **Software Programs Commonly Used to Analyze and Apply Sequence Data**

Software	Name	Application
BLAST	Basic Local Alignment Search Tool	Compares an input sequence with all sequences in a selected database
GRAIL	Gene Recognition and Assembly Internet Link	Finds gene-coding regions in DNA sequences
FASTA	FAST-All derived from FAST-P (protein) and FAST-N (nucleotide) search algorithms	Rapid alignment of pairs of sequences by sequence patterns rather than individual nucleotides
Phred	Phred	Reads bases from original trace data and recalls the bases, assigning quality values to each base

Continued on following page

Table 10.2 **Software Programs Commonly Used to Analyze and Apply Sequence Data** *(continued)*

Software	Name	Application
Polyphred	Polyphred	Identifies single nucleotide polymorphisms (SNPs) among the traces and assigns a rank indicating how well the trace at a site matches the expected pattern for an SNP
Phrap	Phragment Assembly Program	Uses user-supplied and internally computed data quality information to improve accuracy of assembly in the presence of repeats
TIGR Assembler	The Institute for Genomic Research	Assembly tool developed by TIGR to build a consensus sequence from smaller-sequence fragments
Factura	Factura	Identifies sequence features such as flanking vector sequences, restriction sites, and ambiguities.
SeqScape	SeqScape	Mutation and SNP detection and analysis, pathogen subtyping, allele identification, and sequence confirmation
Assign	Assign	Allele identification software for haplotyping
Matchmaker	Matchmaker	Allele identification software for haplotyping

the 3′ end of the primer, DNA polymerase extends the primer. Pyrophosphate (PPi) is released with the formation of the phosphodiester bond between the dNTP and the primer. The PPi is converted to ATP by sulfurylase in the presence of APS. The ATP is used to generate a luminescent signal by luciferase-catalyzed conversion of luciferin to oxyluciferin. The process is repeated with each of the four nucleotides again added sequentially to the reaction. The generation of a signal indicates which nucleotide is the next correct base in the sequence. Results from a pyrosequencing reaction consist of single peaks of luminescence associated with the addition of the complementary nucleotide. If a sequence contains a repeated nucleotide, for instance, GTTAC, the results would be: dG peak, dT peak (double the height of the dG peak), dA peak, dC peak.

Pyrosequencing is most useful for short- to moderate-sequence analysis. It is therefore used mostly for mutation or single nucleotide polymorphism (SNP) detection and typing rather than for generating new sequences. It has been used for applications in infectious disease typing [9,10] and HLA typing.[11]

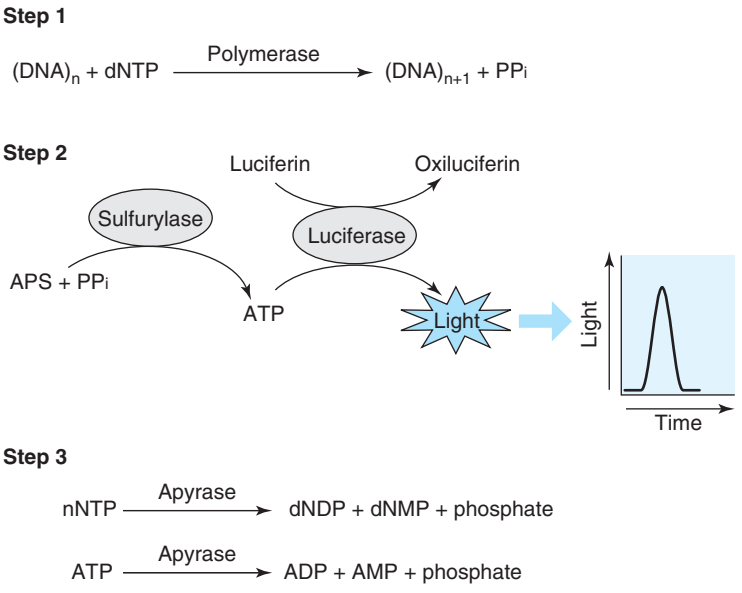
Advanced Concepts

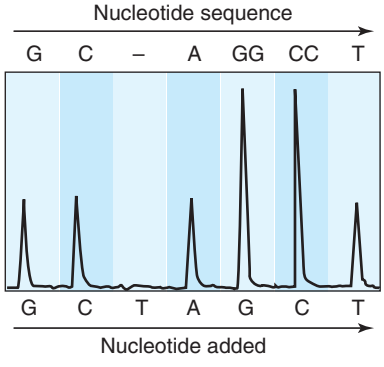
Pyrosequencing requires a single-stranded sequencing template. Methods using streptavidin-conjugated beads have been devised to easily prepare the template. First the region of DNA to be sequenced is PCR-amplified with one of the PCR primers covalently attached to biotin. The amplicons are then immobilized onto the beads and the nonbiotinylated strand denatured with NaOH. After several washings to remove all other reaction components, the sequencing primer is added and annealed to the pure single-stranded DNA template.

Bisulfite DNA Sequencing

Bisulfite DNA sequencing, or methylation-specific sequencing, is a modification of chain termination sequencing designed to detect methylated nucleotides.[12,13] Methylation of cytosine residues in DNA is an important part of regulation of gene expression and chromatin structure (see Chapter 2). Methylated DNA is also involved in cell differentiation and is implicated in a number of diseases, including several types of cancer.

For bisulfite sequencing, 2–4 μg of genomic DNA is cut with restriction enzymes to facilitate denaturation. The enzymes should not cut within the region to be sequenced. The restriction digestion products are resolved on an agarose gel, and the fragments of the size of inter-





■ Figure 10-15 Pyrosequencing is analysis of pyrophosphate (PPi) released when a nucleotide base (dNTP) is incorporated into DNA (top left). The released PPi is a cofactor for ATP generation from adenosine 5′ phosphosulfate (APS). Luciferase plus ATP converts luciferin to oxyluciferin with the production of light which is detected by a luminometer. The system is regenerated with apyrase, that degrades residual free dNTP and dATP (Step 3). As nucleotides are added to the system one at a time, the sequence is determined by which of the four nucleotides generates a light signal.

est are purified from the gel. The purified fragments are denatured with heat (97°C for 5 minutes) and exposed to bisulfite solution (sodium bisulfite, NaOH and hydroquinone) for 16–20 hours. During this incubation, the cytosines in the reaction are deaminated, converting them to uracils, whereas the 5-methyl cytosines are unchanged.

After the reaction, the treated template is cleaned, precipitated, and resuspended for use as a template for PCR amplification. The PCR amplicons are then sequenced in a standard chain termination method. Methylation is detected by comparing the treated sequence with an untreated sequence and noting where in the treated sequence C/G base pairs are not changed to U/G; that is, the sequence will be altered relative to controls at the unmethylated C residues (Fig. 10-16).

Nonsequencing detection methods have also been devised to detect DNA methylation, such as using restriction enzymes to detect restriction sites generated or destroyed by the C>U changes. Other methods use PCR primers that will bind only to the converted or nonconverted sequences so that the presence or absence of PCR product indicates the methylation status. These methods, however, are not always applicable to detection of methylation in unexplored sequences. As the role

Methylated sequence: …GTC^{Me}AGC^{Me}TATCTATC^{Me}GTGCA…
Treated sequence: …GTC^{Me}AGC^{Me}TATUTATC^{Me}GTGUA…

Untreated reference: …GTCAGCTATCTATCGTGCA…
Treated reference: …GTUAGUTATUTATUGTGUA…

Figure 10-16 Exposure of a sequence (top) to bisulfite will result in conversion of unmethylated cytosines to uracils (treated sequence). By comparing the sequence treated with bisulfite to mock treated reference sequence, the methylated cytosines will become apparent as they are not changed to uracil (U) by the bisulfite.

of methylation and epigenetics in human disease is increasingly recognized, bisulfite sequencing has become a popular method in the research laboratory. To date, however, this method has had limited use in clinical analysis.

Bioinformatics

Information technology has had to encompass the vast amount of data arising from the growing numbers of sequence discovery methods, especially direct sequencing and array technology. This deluge of information requires careful storage, organization, and indexing of large amounts of data. **Bioinformatics** is the merger of biology with information technology. Part of the practice in this field is biological analysis **in silico**; that is, by computer rather than in the laboratory. Bioinformatics dedicated specifically to handling sequence information is sometimes termed **computational biology**. A list of some of the terms used in bioinformatics is shown in Table 10.3. The handling of the mountains of data being generated requires continual renewal of stored data and a number of databases are available for this purpose.[14]

Table 10.3 Bioinformatics Terminology

Term	Definition
Identity	The extent to which two sequences are the same
Alignment	Lining up two or more sequences to search for the maximal regions of identity in order to assess the extent of biological relatedness or homology
Local alignment	Alignment of some portion of two sequences
Multiple sequence alignment	Alignment of three or more sequences arranged with gaps so that common residues are aligned together
Optimal alignment	The alignment of two sequences with the best degree of identity
Conservation	Specific sequence changes (usually protein sequence) that maintain the properties of the original sequence
Similarity	The relatedness of sequences, the percent identity or conservation
Algorithm	A fixed set of commands in a computer program
Domain	A discreet portion of a protein or DNA sequence
Motif	A highly conserved short region in protein domains
Gap	A space introduced in alignment to compensate for insertions or deletions in one of the sequences being compared
Homology	Similarity attributed to descent from a common ancestor
Orthology	Homology in different species due to a common ancestral gene
Paralogy	Homology within the same species resulting from gene duplication
Query	The sequence presented for comparison with all other sequences in a selected database
Annotation	Description of functional structures, such as introns or exons in DNA or secondary structure or functional regions to protein sequences
Interface	The point of meeting between a computer and an external entity, such as an operator, a peripheral device, or a communications medium
GenBank	The genetic sequence database sponsored by the National Institutes of Health
PubMed	Search service sponsored by the National Library of Medicine that provides access to literature citations in Medline and related databases
SwissProt	Protein database sponsored by the Medical Research Council (United Kingdom)

Standard expression of sequence data is important for the clear communication and organized storage of sequence data. In some cases, such as in heterozygous mutations, there may be more than one base or mixed bases at the same position in the sequence. Polymorphic or heterozygous sequences are written as **consensus sequences**, or a family of sequences with proportional representation of the polymorphic bases. The International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology (IUB) have assigned a universal nomenclature for mixed, degenerate, or wobble bases (Table 10.4). The base designations in the IUB code are used to communicate consensus sequences and for computer input of polymorphic sequence data.

The Human Genome Project

From the first description of its double helical structure in 1953 to the creation of the first recombinant molecule in the laboratory in 1972, DNA and the chemical nature of the arrangement of its nucleotides have attracted interest. Gradually, this information began to accumulate, first regarding simple microorganisms and then partially in lower and higher eukaryotes. The deciphering of the human genome is a hallmark of molecular biology. It is a benchmark in the ongoing discovery of the molecular basis for disease and the groundwork of molecular diagnostics. In the process of solving the human DNA sequence, genomes of a variety of clinically significant organisms have also been deciphered, advancing typing and predicting infectious disease treatment outcomes.

Table 10.4 **IUB Universal Nomenclature for Mixed Bases**

Symbol	Bases	Mnemonic
A	Adenine	Adenine
C	Cytosine	Cytosine
G	Guanine	Guanine
T	Thymine	Thymine
U	Uracil	Uracil
R	A, G	puRine
Y	C, T	pYrimidine
M	A, C	aMino
K	G, T	Keto
S	C, G	Strong (3 H bonds)
W	A, T	Weak (2 H bonds)
H	A, C, T	Not G
B	C, G, T	Not A
V	A, C, G	Not T
D	A, G, T	Not C
N	A, C, G, T	aNy
X, ?	Unknown	A or C or G or T
O, -	Deletion	

The first complete genome sequence of a clinically important organism was that of Epstein-Barr virus published in 1984.[15] The 170,000–base pair sequence was determined using the M13 template preparation/chain termination manual sequencing method. In 1985 and 1986 the possibility of mapping or sequencing the human genome was discussed at meetings at the University of California, Santa Cruz; Cold Spring Harbor, New York; and at the Department of Energy in Santa Fe, New Mexico. The idea was controversial because the two to five billion dollar cost of the project might not justify the information gained, most of which would be sequences of "junk," or non–gene-coding DNA. Furthermore, there was no available technology up to the massive task. The sequencing automation and the computer power necessary to assemble the three billion bases of the human genome into an organized sequence of 23 chromosomes was not yet developed.

Nevertheless, several researchers, including Walter Gilbert (of Maxam-Gilbert sequencing), Robert Sinsheimer, Leroy Hood, David Baltimore, David Botstein, Renato Dulbecco, and Charles DeLici, saw that the project was feasible because technology was rapidly advancing toward full automation of the process. In 1982 Akiyoshi Wada had proposed automated sequencing machinery and had gotten support from Hitachi Instruments. In 1987 Smith and Hood announced the first automated DNA sequencing machine.[16] Advances in the chemistry of the sequencing procedure, described in the first sections of this chapter, were accompanied by advances in the biology of DNA mapping, with methods such as pulsed field gel electrophoresis,[17,18] restriction fragment length polymorphism analysis,[19] and transcript identification.[20] Methods were developed to clone large (500 kbp) DNA fragments in artificial chromosomes, providing long contiguous sequencing templates.[21] Finally, application of capillary electrophoresis to DNA resolution[22–24] made the sequencing procedure even more rapid and cost-efficient.

Table 10.5 Model Organisms Sequenced During the Human Genome Project

Organism	Genome Size (Mb)	Estimated Number of Genes
Epstein-Barr virus	0.17	80
Mycoplasma genitalium	0.58	470
Haemophilus influenzae	1.8	1740
Escherichia coli K-12	4.6	4377
E. coli O157	5.4	5416
Saccharomyces cerevisiae	12.5	5770
Drosophila melanogaster	180	13,000
Caenorhabditis elegans	97	19,000
Arabidopsis thaliana	100	25,000

With these advances in technology, the Human Genome Project was endorsed by the National Research Council. The National Institutes of Health (NIH) established the Office of Human Genome Research with James Watson as its head. Over the next 5 years, meetings on policy, ethics, and cost of the project resulted in a plan to complete 20 Mb of sequence of model organisms by 2005 (Table 10.5). To organize and compare the growing amount of sequence data, the Basic Local Alignment Search Tool and Gene Recognition and Assembly Internet Link algorithms were introduced in 1990.[25,26]

For the human sequence, the decision was made to use a composite template from multiple individuals rather than a single genome from one donor. Human DNA was donated by 100 anonymous volunteers; only 10 of these genomes were sequenced. Not even the volunteers knew if their DNA was used for the project. To ensure accurate and high-quality sequencing, all regions were sequenced 5–10 times over.

Another project started with the same goal. In 1992 Craig Venter left the NIH to start The Institute for Genomic Research (TIGR). Venter's group completed the first sequence of a free-living organism (*Haemophilus influenzae*)[27] and the sequence of the smallest free-living organism (*Mycoplasma genitalium*).[28] Venter established a new company named Celera and proposed to complete the human genome sequence in 3 years for $300 million, faster and cheaper than the NIH project. Meanwhile, Watson had resigned as head of the NIH project and was replaced by Francis Collins. In response, the Wellcome Trust doubled its support of the NIH project. The NIH moved its completion date from 2005 to 2003, with a working draft to be completed by 2001. Thus began a competitive effort to sequence the human genome on two fronts.

The two projects approached the sequencing differently (Fig. 10-17). The NIH method (**hierarchal shotgun sequencing**) was to start with sequences of known

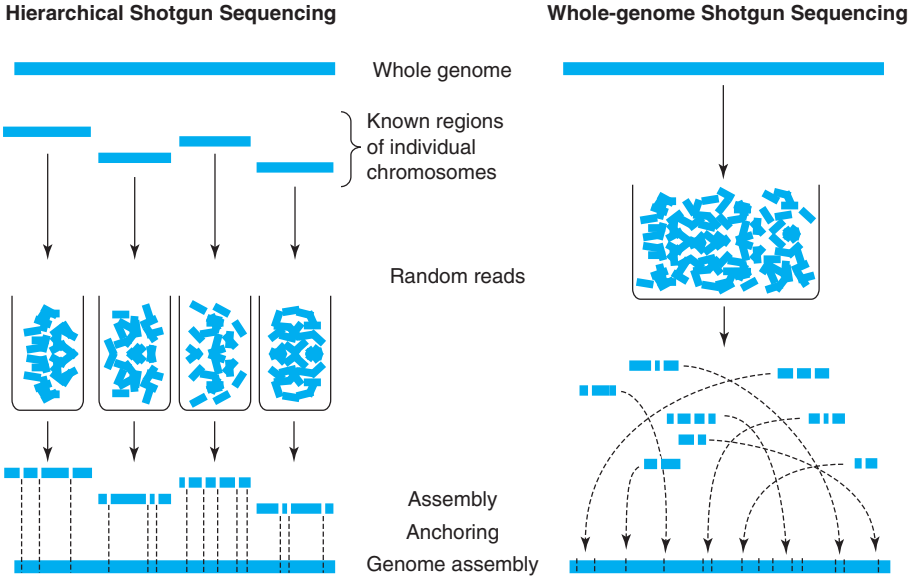


Figure 10-17 Comparison of two approaches for sequencing of the human genome. The hierarchal shotgun approach taken by the NIH (left) was to sequence from known regions so that new sequences could easily be located in the genome. The Celera whole-genome shotgun approach (right) was to sequence random fragments from the entire genome and then to assemble the complete sequence with computers.

regions in the genome and "walk" further away into the chromosomes, always aware of where the newly generated sequences belonged in the human genome map. The researchers at Celera had a different idea. Their approach (**whole genome shotgun sequencing**) was to start with 10 equivalents of the human genome cut into small fragments and randomly sequence the lot. Then, powerful computers would find overlapping sequences and use those to assemble the billions of bases of sequence into their proper chromosomal locations.

Initially, the Celera approach was met with skepticism. The human genome contains large amounts of repeated sequences (see Chapter 11), some of which are very difficult to sequence and even more difficult to map properly. A random sequencing method would repeatedly cover areas of the genome that are more easily sequenced and miss more difficult regions. Moreover, assembly of the whole sequence from scratch with no chromosomal landmarks would take a prohibitive amount of computer power. Nonetheless, Celera began to make headway (some alleged with the help of the publicly published sequences from the NIH), and eventually the NIH project modified its approach to include both methods.

Over the next months, some efforts were made toward combining the two projects, but these efforts broke down over disagreements over database policy and release of completed sequences. The result of the competition was that the rough draft of the sequence was completed by both projects earlier than either group had proposed, in June 2000. A joint announcement was made, and both groups published their versions of the genome, the NIH version in the journal *Nature*[29] and the Celera version in the journal *Science*.[30]

The sequence completed in 2000 was a rough draft of the genome; that is, there were still areas of missing sequence and sequences yet to be placed. Only chromosomes 21 and 22, the smallest of the chromosomes, had been fully completed. In the ensuing years, the finished sequences of each chromosome are being released (Table 10.6).

Even with the rough draft, interesting characteristics of the human genome were revealed. The size of the entire genome is 2.91Gbp (2.91 billion base pairs). The genome was initially calculated as 54% AT, 38% GC with 8% of the bases still to be determined. Chromosome 2 is the most GC-rich chromosome (66%), and chromosome X has the fewest GC base pairs (25%). A most surprising discovery was that the number of genes, estimated to be from 20,000 to 30,000, was much lower than expected. The average size of a human gene is 27 kbp. Chromosome 19 is the most gene-rich per unit length (23 genes/Mbp). Chromosomes 13 and Y have the fewest genes per base pair (5 genes/Mbp). Only about 2% of the sequences code for genes; 30%–40% of the genome consists of repeat sequences. There is one SNP between two random individuals found approximately every 1000 bases along the human DNA sequence. More detailed information, databases, references, and updated information are available at ncbi.nlm.nih.gov

Table 10.6 **Completed Chromosomes**

Chromosome	Completion Date
21	December 1999
22	May 2000
20	December 2001
14	January 2003
Y	June 2003
7	July 2003
6	October 2003
13	March 2004
19	March 2004
10	May 2004
9	May 2004
5	September 2004
16	December 2004
X	March 2005
2	April 2005
4	April 2005
8	January 2006
11	March 2006
12	March 2006
17	April 2006
3	April 2006
1	May 2006

The significance of the Human Genome Project to diagnostics can be appreciated with the example of the discovery of the gene involved in cystic fibrosis. Seven years of work were required for discovery of this gene. With proper mapping information, a gene for any condition can now be found by computer, already sequenced, in a matter of minutes. Of course, all genetic diseases are not due to malfunction of a single gene. In fact, most diseases and normal states are driven by a combination of genes as well as by environmental influences. Without

the rich information afforded by the sequence of the human genome, identification of these multicomponent diseases would be almost impossible.

Another project has been launched to further define the relationship between gene sequence and disease. This is the Human Haplotype Mapping, or HapMap, Project. The goal of this project is to find blocks of sequences that are inherited together, marking particular traits and possibly disease-associated genetic lesions. A description of this project is presented in Chapter 11.

The technology developed as part of the Human Genome Project has made sequencing a routine method used in the clinical laboratory. Small, cost-effective sequencers are available for rapid sequencing, methods that were not practical only a few years ago. In the clinical laboratory, sequencing is actually **resequencing**, or repeated analysis of the same sequence region, to detect mutations or to type microorganisms, making the task even more routine. The technology continues to develop, to reduce the cost and labor of sequencing larger and larger areas, so that several regions can be sequenced to detect multicomponent diseases or to predict predisposition to disease. Accurate and comprehensive sequence analysis is one of the most promising areas of molecular diagnostics.

• STUDY QUESTIONS •

1. Read 5′ to 3′ the first 20 bases of the sequence in the gel on the right in Figure 10-8.

2. After an automated dye primer sequencing run, the electropherogram displays consecutive peaks of the following colors:
   red, red, black, green, green, blue, black, red, green, black, blue, blue, blue
   If the computer software displays the fluors from ddATP as green, ddCTP as blue, ddGTP as black, and ddTTP as red, what is the sequence of the region given?

3. After an automated dye terminator sequencing run, the electropherogram displays bright (high, wide) peaks of fluorescence, obliterating some of the sequencing peaks. What is the most likely cause of this observation? How might it be corrected?

4. In a manual sequencing reaction, the DNA ladder on the polyacrylamide gel is very bright and readable at the bottom of the gel, but the larger (slower-migrating) fragments higher up are very faint. What is the most likely cause of this observation? How might it be corrected?

5. In an analysis of the *p53* gene for mutations, the following sequences were produced. For each sequence, write the expected sequence of the opposite strand that would confirm the presence of the mutations detected.
   Normal:
   5′TATCTGTTCACTTGTGCCCT3′
   (Homozygous substitution)
   5′TATCTGTTCATTTGTGCCCT3′
   (Heterozygous substitution)
   5′TATCTGT(T/G)CACTTGTGCCCT3′
   (Heterozygous Deletion)
   5′TATCTGTT(C/A)(A/C)(C/T)T(T/G)(G/T)(T/G)(G/C)CC(C/T)(T/…3′

6. A sequence, TTGCTGCGCTAAA, may be methylated at one or more of the cytosine residues: After bisulfite sequencing, the following results are obtained:
   Bisulfite treated: TTGCTGTGCTAAA
   Untreated: TTGCTGCGCTAAA
   Write the sequences showing the methylated cytosines as C^{Me}.

7. In a pyrosequencing read out, the graph shows peaks of lumninescence corresponding to the addition of the following nucleotides:
   dT peak, dC peak (double height), dT peak, dA peak
   What is the sequence?

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SECTION 3

Techniques in the Clinical Lab

Chapter 11

Lela Buckingham

DNA Polymorphisms and Human Identification

OUTLINE

OBJECTIVES

- Compare and contrast different types of polymorphisms.
- Define restriction fragment length polymorphisms and discuss how they are used in genetic mapping, parentage testing, and human identification.
- Describe short tandem repeat structure and nomenclature.
- Describe gender identification using the amelogenin locus.
- Explain matching probabilities and the contribution of allele frequencies to the certainty of matching.
- Describe the use of Y-STR in forensic and lineage studies.
- Give examples of the use of STR for bone marrow engraftment monitoring.
- Show how STR may be used for quality assurance of histological sections.
- Define single nucleotide polymorphisms and their potential use in disease gene mapping.
- Discuss mitochondrial DNA typing.

As discussed in Chapter 8, polymorphisms are DNA sequences that differ from the sequences of a majority of a population but are still shared by a certain percentage. These sequences can be as small as a single base pair or involve thousands of base pairs.

Types of Polymorphisms

The probability of polymorphic DNA in humans is great due to the relatively large size of our genome, 98% of which does not code for genes. At the nucleotide-sequence level, it is estimated that genome sequences differ by one nucleotide every 1000–1500 bases. These single nucleotide differences, or **single nucleotide polymorphisms** (SNPs), may occur in gene-coding regions as well as intergenic sequences (see Chapter 4 for the nature of the genetic code and Chapter 8 for a discussion of silent and conservative mutations in coding regions).

The human leukocyte antigen (HLA) locus is a familiar example of a highly polymorphic region of human DNA where single nucleotide changes occur more frequently. The variable nucleotide sequences in this locus code for peptides that establish self-identity of the immune system. The extent of similarity or compatibility between immune systems of transplant recipients and potential donors can thus be determined by comparing DNA sequences (see Chapter 15). HLA typing may also be used for exclusion in human identification tests.

Some human sequence polymorphisms affect many base pairs. Large blocks of repeated sequences may be inverted, deleted, or duplicated from one individual to another. **Long interspersed nucleotide sequences** (LINES) are highly repeated sequences, 6–8 kbp in length, that contain RNA polymerase promoters and open reading frames related to the reverse transcriptase of retroviruses. There are more than 500,000 of these LINE-1 (L1) elements, making up more than 15% of the human genome. There are even more **short interspersed nucleotide sequences** (SINES) scattered over the genome. SINES, 0.3 kbp in size, are present in over 1,000,000 copies per genome. SINES include **Alu elements**, named for harboring recognition sites for the *Alu*I restriction enzyme. LINES and SINES are also known as mobile elements or transposable elements. They are copied and spread by recombination and reverse transcription and may be responsible for formation of **pseudogenes** (intronless, nonfunctional copies of active genes) throughout the human genome. Shorter blocks of repeated sequences also undergo expansion or shrinkage through generations. Examples of the latter are **short tandem repeats** (STRs) and **variable number tandem repeats** (VNTRs).

Single nucleotide polymorphisms, larger sequence variants, and tandem repeats can be detected by observing changes in the restriction map of a DNA region. Analysis of restriction fragments by Southern blot reveals **restriction fragment length polymorphisms** (RFLPs). Particular types of polymorphisms, specifically SNPs, VNTRs, STRs, and RFLPs, are routinely used in the laboratory (Table 11.1).

RFLP Typing

The first polymorphic RFLP was described in 1980. RFLPs were the original molecular targets used for gene mapping, human identification, and parentage testing. RFLPs are observed as differences in the sizes and number of fragments generated by restriction enzyme digestion of DNA (Fig. 11-1). Fragment sizes may vary as a result of changes in the nucleotide sequence in or between the recognition sites of a restriction enzyme. Nucleotide changes can destroy, change, or create restriction enzyme sites, altering the number of fragments.

The first step in using RFLPs is to construct a restriction enzyme map of the DNA region under investigation. (Construction of restriction maps is described in Chapter

Table 11.1 **Types of Useful Polymorphisms and Laboratory Methods**

Polymorphism	Structure	Detection Method
RFLP	One or more nucleotide changes that affect the size of restriction enzyme products	Southern blot
VNTR	Repeats of 10–50 base sequences in tandem	Southern blot, PCR
STR	Repeats of 1–10 base sequences in tandem	PCR
SNP	Alterations of a single nucleotide	Sequencing, other

Figure 11-1 Types of DNA sequence alterations that change restriction fragment lengths. The normal sequence (top) has an *Eco* R1 site (GAATTC). Single base changes (point mutations, second line) can destroy the *Eco*R1 site or create a new restriction site, as can insertions, duplications, or deletions of any number of bases (third through fifth lines). Insertions, duplications, and deletions between two restriction sites change fragment size without affecting the restriction sites themselves.

6.) Once the restriction map is known, the number and sizes of the restriction fragments of a test DNA region cut with restriction enzymes are compared with the number and sizes of fragments expected based on the restriction map. Polymorphisms are detected by observing fragment numbers and sizes different from those expected from the reference restriction map. An example of a polymorphism in a restriction site is shown in Figure 11-2. In a theoretical linear piece of DNA, loss of the recognition site for the enzyme (*Bgl*II in the figure) results in alteration of the size and number of bands detected after gel electrophoresis.

Initially, RFLP typing in humans required the use of the Southern blot technique (see Chapter 5). DNA was cut with restriction enzymes, resolved by gel electrophoresis, and blotted to a membrane. Probes to specific regions of DNA containing potential RFLPs were then hybridized to the DNA on the membrane to determine the size of the resulting bands. In Figure 11-3, the pattern of bands resulting from a Southern blot analysis of RFLP is shown. Note that not all of the restriction fragments are detected by the probe; yet the three polymorphisms can still be identified.

DNA is inherited as one chromosome complement from each parent. Each chromosome carries its polymor-

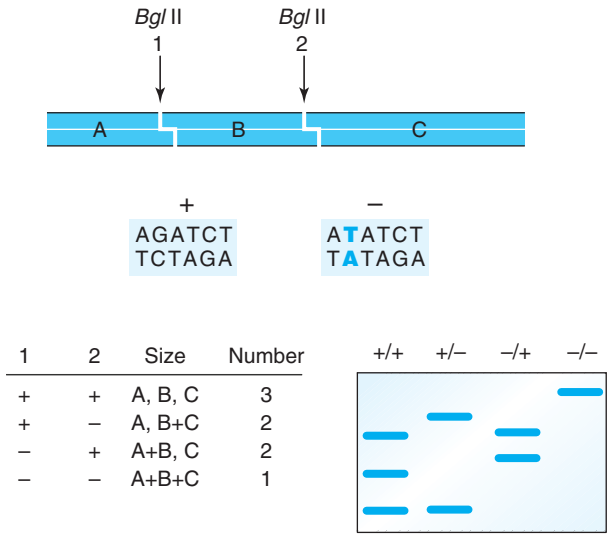


Figure 11-2 A linear piece of DNA with two polymorphic *Bgl* II restriction enzyme sites, designated here as 1 and 2, will yield different fragment sizes, depending on the presence of neither, either, or both of the restriction sites. For instance a G→T mutation will change the sequence of the normal site (+) to one not recognized by the enzyme (–). The presence or absence of the polymorphic sites is evident from the number and size of the fragments after cutting the DNA with *Bgl* II (bottom right).

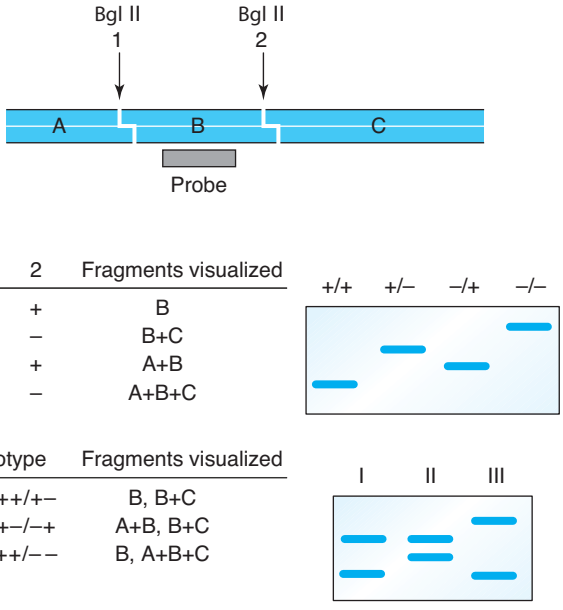


Figure 11-3 Using a Southern blot to probe for RFLP. With the same region shown in Figure 11-2, only the fragments with complementary sequences to a probe to the B region (top) can be visualized.

phisms so that the offspring inherits a combination of the parental polymorphisms. When visualized as fragments that hybridize to a probe of a polymorphic region, the band patterns represent the combination of RFLPs inherited from each parent. Due to recombination and random assortment, each person has a unique set of RFLPs, half inherited maternally and half paternally. Every genotype will yield a descriptive band pattern as shown in Figure 11-3.

Over many generations, mutations, intra- and interchromosomal recombination, gene conversion, and other genetic events have increased the diversity of DNA sequences. One consequence of this genetic diversity is that a single **locus,** that is, a gene or region of DNA, will have several versions, or **alleles**. Human beings are diploid; that is, people have two copies of every locus. In other words, each person has two alleles of each locus. If these alleles are the same, the locus is **homozygous**; if the two alleles are different, the locus is **heterozygous**.

Depending on the extent of diversity or polymorphism of a locus, any two people can share the same alleles or have different alleles. More closely related individuals are likely to share more alleles than unrelated persons. In the examples shown in Figure 11-3, (+ +), (+ −), (− +), and (− −) describe the presence (+) or absence (−) of *Bgl*II sites making up four alleles of the locus detectable by Southern blot. In the illustration, genotypes I and II both have the (+ −) allele on one chromosome, but genotype I has (+ +), and genotype II has (− +) on the other chromosome. This appears in the Southern blot results as one band of equal size between the two genotypes and one band that is a different size. Two individuals can share both alleles at a single locus, but the chances of two individuals, except for identical twins, sharing the same alleles decrease 10-fold with each additional locus tested.[1]

More than 2000 RFLP loci have been described in human DNA. The uniqueness of the collection of polymorphisms in each individual is the basis for human identification at the molecular level. Detection of RFLPs by Southern blot made positive paternity testing and human identification possible for the first time. RFLP protocols for human identification in most North American laboratories used the restriction enzyme *Hae*III for fragmentation of genomic DNA. Many European laboratories used the *Hinf*I enzyme. These enzymes cut DNA frequently enough to reveal polymorphisms in multiple locations throughout the genome. To regulate results from independent laboratories, the Standard Reference Material (SRM) DNA Profiling Standard for RFLP analysis was released in 1992. The SRM supplies cell pellets, genomic DNA, gel standards, precut DNA, electrophoresis materials, molecular weight markers, and certified values for final analysis. These materials were designed to maintain reproducibility of the RFLP process across laboratories.

Genetic Mapping With RFLPs

Polymorphisms are inherited in a mendelian fashion, and locations of many polymorphisms in the genome are known. Therefore, polymorphisms can be used as landmarks, or **markers**, in the genome to determine the location of other genes. In addition to showing clear family history or direct identification of a genetic factor, one can confirm that a disease has a genetic component by demonstrating a close genetic association or linkage to a known marker. Formal statistical methods are used to determine the probability that an unknown gene is located close to a known marker in the genome. The more frequently a particular polymorphism is present in persons with a disease phenotype, the more likely the affected gene is located

Historical Highlights

Mary Claire King used RFLP to map one of the genes mutated in inherited breast cancer.[61,62] Following extended families with high incidence of breast and ovarian cancer, she found particular RFLP always present in affected family members. Because the location in the genome of the RFLP was known (17q21), the *BRCA1* gene was thereby mapped to this position on the long arm of chromosome 17.

close to the polymorphism. This is the basis for linkage mapping and one of the ways genetic components of disease are identified.

RFLP and Parentage Testing

In diploid organisms, chromosomal content is inherited half from each parent. This includes the DNA polymorphisms located throughout the genome. Taking advantage of the unique combination of RFLP in each individual, one can infer a parent's contribution of alleles to a son or daughter from the combination of alleles in the child and those of the other parent. The fragment sizes of an individual as a combination of those from each parent is illustrated in Figure 11-4. In a paternity test, the alleles or fragment sizes of the offspring and the mother are analyzed. The remaining fragments (the ones that do not match the mother) have to come from the father. Alleged fathers are identified, or **included**, based on the ability to provide the remaining alleles. Aside from possible mutations, a difference in just one allele may exclude paternity.

A simplified RFLP paternity test is shown in Figure 11-5. Of the two alleged fathers shown, only one could supply the fragments not supplied by the mother. In this example, only two loci are shown. A parentage test requires analysis of at least eight loci. The more loci tested, the higher the probability of positive identification of the father.

Human Identification Using RFLP

The first genetic tool used for human identification was the ABO blood group antigens. Although this type of

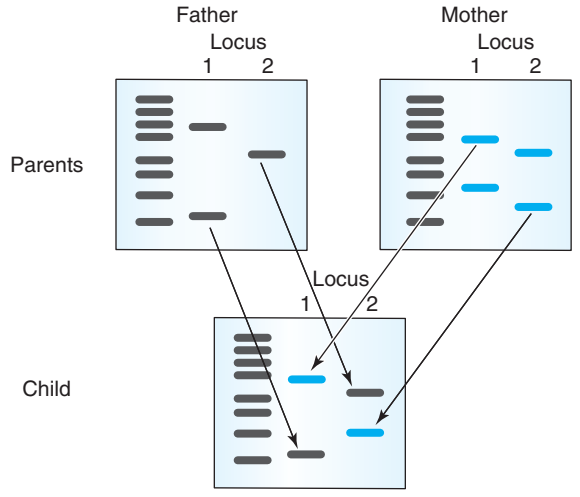


Figure 11-4 RFLP inheritance. Two different genetic regions, or loci, are shown, locus 1 and locus 2. There are several versions or alleles of each locus. Note that the father is heterozygous at locus 1 and homozygous at locus 2. The alleles in the child will be a combination of one allele from each parent.

analysis could be performed in a few minutes, the discrimination power was low. With only four possible groups, this method was only good for **exclusion** (elimination) of a person as a source of biological material and

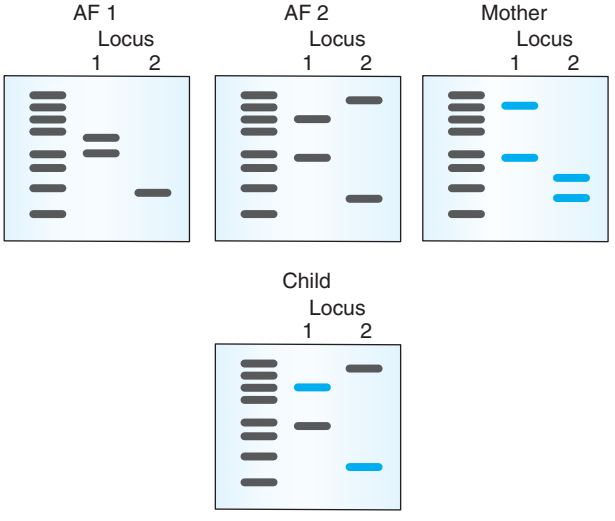


Figure 11-5 Two alleged fathers (AF) are being tested for paternity of the child whose partial RFLP profile is shown in the bottom gel. The mother's alleles are shown in green. One AF (AF1) is excluded from paternity.

was informative only in 15% to 20% of cases. Analysis of the polymorphic HLA loci could add a higher level of discrimination, with exclusion in 90% of cases. Testing both ABO and HLA could exclude a person in 97% of cases but still did not provide positive identification.

The initial use of DNA as an identification tool relied on RFLP detectable by Southern blot. As shown in Figure 11-1, RFLP can arise from a number of genetic events. One of these is the insertion or deletion of nucleotides between the restriction sites. This occurs frequently in repeated sequences in DNA. Tandem repeats of sequences of all sizes are present in genomic DNA (Fig. 11-6). Repeats of eight or more nucleotides are called **variable number tandem repeats** (VNTRs), or **minisatellites**. These repeats are large enough so that loss or gain of one repeat can be resolved by gel electrophoresis of a restriction enzyme digest. The frequent cutters, *Hae*III (recognition site GGCC) or *Hinf*I (recognition site GANTC), generate fragments that are small enough to resolve those that contain different numbers of repeats and thereby give an informative pattern by Southern blot.

The first human DNA profiling system was introduced by the United Kingdom Forensic Science Service in 1985 using Alec Jeffreys' Southern blot **multiple locus probe** (MLP)-RFLP system.[2] This method utilized three to five probes to analyze three to five loci on the same blot. Results of probing multiple loci at once produced patterns that were highly variable between individuals but that required some expertise to optimize and interpret. In 1990, **single locus probe** (SLP) systems were established in Europe and North America.[3,4] Analysis of one locus at a time yielded simpler patterns, which were much easier to interpret, especially in cases where specimens might contain DNA from more than one individual (Fig 11-7).

The RFLP Southern blot technique required 100 ng–1 µg of relatively high quality DNA, 1–20 kbp in size.

Historical Highlights

Professor Sir Alec John Jeffreys, a British geneticist, first developed techniques for **genetic profiling**, or **DNA fingerprinting**, using RFLP to identify humans. The technique has been applied to forensics and law enforcement to resolve paternity and immigration disputes and can be applied to nonhuman species, for example in wildlife population genetics. The initial application of this DNA technique was in a regional screen of human DNA to identify the rapist and killer of two girls in Leicestershire, England, in 1983 and 1986. Colin Pitchfork was identified and convicted of murder after samples taken from him matched semen samples taken from the two dead girls.

Furthermore, large, fragile 0.7% gels were required to achieve adequate band resolution, and the ^{32}P-based probe system could take 5–7 days to yield clear results. After visually inspecting the band patterns, profiles were subjected to computer analysis to accurately size the restriction fragments and apply the results to an established matching criterion. RFLP is an example of a **continuous allele system** in which the sizes of the fragments define alleles. Therefore, precise band sizing was critical to the accuracy of the results. A match implied inclusion, which was refined by determination of the genotype frequency of each allele in the general or local population. This process established likelihood of the same genotype occurring by chance. The probability of two people having the same set of RFLP, or **profile**, becomes lower and lower as more loci are analyzed.

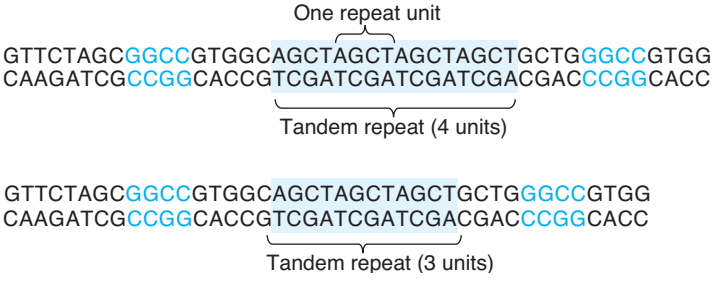


Figure 11-6 A tandem repeat is a direct repeat 1 to >100 nucleotides in length. The one shown has a 4-bp repeat unit. A gain or loss of repeat units forms a new allele. New alleles can be detected as variations in fragment size on digestion with *Hae* III (green recognition sites).

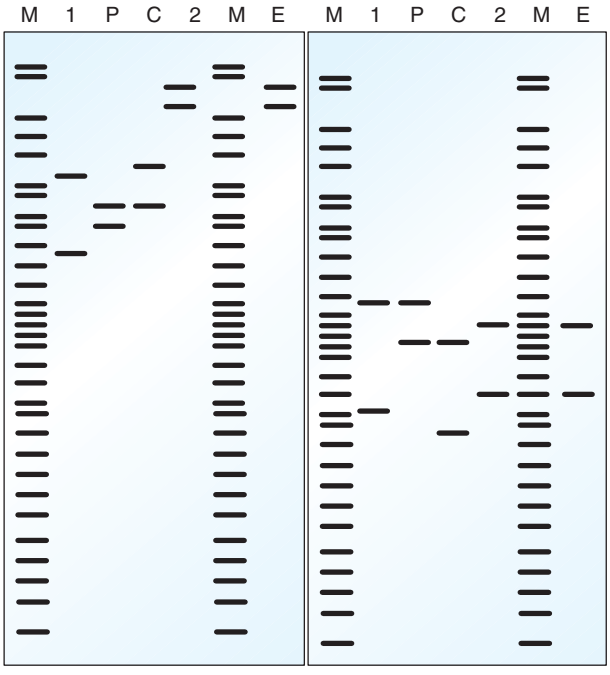


Figure 11-7 Example of RFLP crime evidence using two single-locus probes. M are molecular weight markers, 1 and 2 are suspects. C is the child victim, and P is the parent of the child victim. E is evidence from the crime scene. For both loci probed, suspect 2 "matches" the evidence found at the crime scene. Positive identification of suspect 2 requires further determination of the frequencies of these specific alleles in the population and the probability of matching them by chance.

STR Typing by PCR

The first commercial and validated PCR-based typing test specifically for forensic use was the HLA DQ alpha system, now called DQA1, developed by Cetus Corporation in 1986.[5] This system could distinguish 28 DQA1 types. With the addition of another commercial system, the Polymarker (PM) system, the analyst could type five additional genetic markers. The PM system is a set of primers complementary to sequences flanking **short tandem repeats** (STRs), or **microsatellites**. STRs are similar to VNTRs but have smaller repeat units of 1–7 base pairs. (The exact repeat unit size limit of STR varies 7–10 bp, depending on different texts and reports.) Because of the increased power of discrimination and ease of use of STR, the HLA DQA forensic DNA amplification and typing kit was discontinued in 2002.

Advanced Concepts

At least three to seven RFLP probes were initially required to determine genetic identity. Available probes included G3, MS1, MS8, MS31, and MS43, which were subclones of Jeffreys' multilocus probes 33.6 and 33.15 and pYNH24m, MS205, and MS621.[55] Single locus probes MS1, MS31, MS43, G3, and YNH24 were used by Cellmark in the O.J. Simpson trial in 1996.

The tandem repeat shown in Figure 11-6 is an STR with a 4 bp repeat unit, AGCT. Occasionally, STRs contain repeat units with altered sequences, or **microvariants**, repeat units missing one or more bases of the repeat. These differences have arisen through mutation or other genetic events.

In contrast to VNTRs, the smaller STRs are efficiently amplified by PCR, easing specimen demands significantly. Long intact DNA fragments are not required to detect the STR products; therefore degraded or otherwise less than optimal specimens are potentially informative. The amount of specimen required for STR analysis by PCR is reduced from 1 μg to 10 ng, a key factor for forensic analysis.[6] Furthermore, PCR procedures shorten the analysis time from several weeks to 24–48 hours. Careful design of primers and amplifications facilitated multiplexing and automation.[7]

STR alleles are identified by PCR product size. Primers are designed to produce amplicons 100–400 bp in which the STRs are embedded (Fig. 11-8). The sizes of the PCR products are influenced by the number of embedded repeats. If one of each primer pair is labeled with a fluorescent marker, the PCR product can be analyzed in fluorescent detection systems. Silver-stained gels may also be used; however, capillary electrophoresis with fluorescent dyes is the preferable method, especially for high throughput requirements.

To perform genotyping, test DNA is mixed with the primer pairs, buffer, and polymerase to amplify the test loci. A control DNA standard is also amplified. Following

Advanced Concepts

Theoretically, the minimal sample requirement for polymerase chain reaction analysis is a single cell. A single cell has approximately 6 pg of DNA. This number is derived from the molecular weight of A/T and G/C base pairs (617 and 618 g/mol, respectively). There are about three billion base pairs in one copy of the human genome; therefore for one genome copy:

3×10^9 bp x 618 g/mol/bp $= 1.85 \times 10^{12}$ g/mol

1.85×10^{12} g/mol $\times$ 1 mol/6.023×10^{23} molecules $= 3.07 \times 10^{-12}$ g $=$ 3pg

A diploid cell has two genome copies, or 6 pg of DNA. One ng (1000 pg) of DNA should, therefore, contain 333 copies (1000 pg/3pg/genome copy) of each locus.

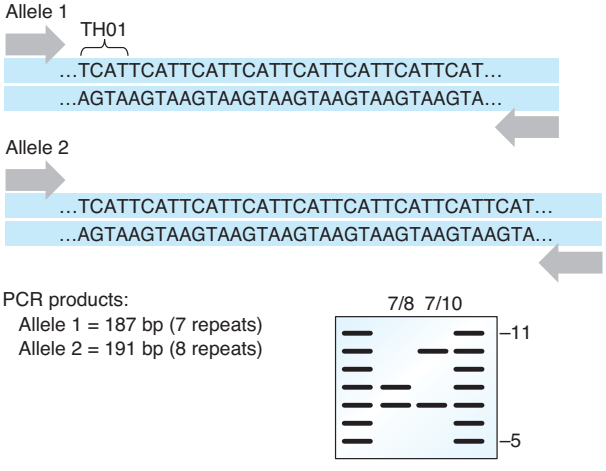


Figure 11-8 Short tandem repeat TH01 (repeat unit TCAT) linked to the human tyrosine hydroxylase gene on chromosome11p15.5. Primers are designed to amplify short regions containing the tandem repeats. Allelic ladders consisting of all alleles in the human population (flanking lanes in the gel shown at bottom right) are used to determine the number of repeats in the locus by the size of the amplicon. The two alleles shown contain 7 and 8 repeats. If these alleles were found in a single individual, that person would be heterozygous for TH01 with a genotype of 7/8.

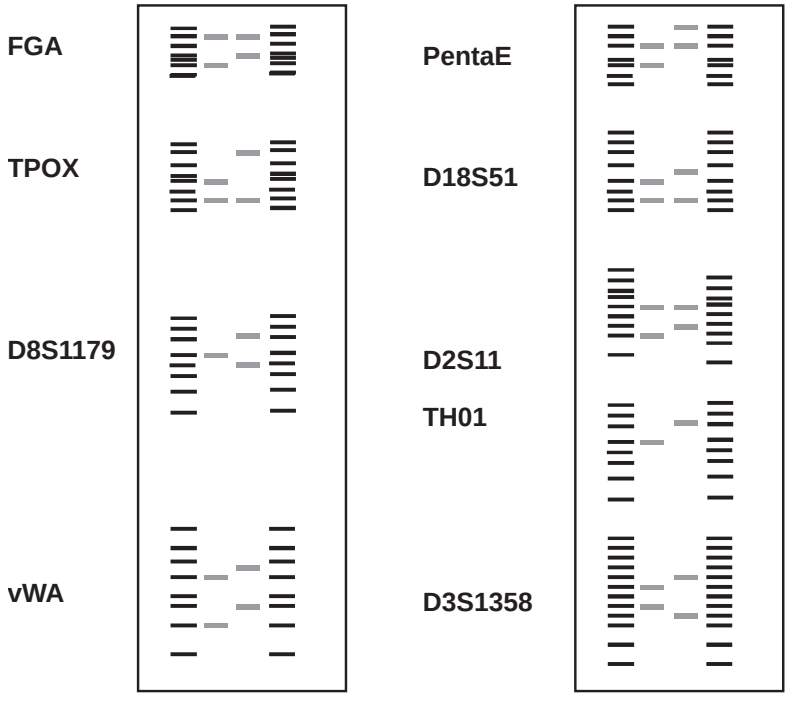
amplification, each sample PCR product is combined with **allelic ladders** (sets of fragments representing all possible alleles of a repeat locus) and **internal size standards** (molecular weight markers) in formamide for electrophoresis. After electrophoresis, detection and analysis software will size and identify the alleles. In contrast to RFLPs and VNTRs, STRs are **discrete allele systems** in which a finite number of alleles are defined by the number of repeat units in the tandem repeat (see Fig. 11-8). Several commercial systems are available consisting of labeled primers for one locus to more than 16 loci. The allelic ladders in these reagent kits allow accurate identification of the sample alleles (Fig. 11-9).

Advances in fluorescence technology have increased the ease and sensitivity of STR allele identification (Fig. 11-10). Although capillary electrophoresis is faster and more automated than gel electrophoresis, a single run through a capillary can resolve only loci whose allele ranges do not overlap. The number of loci that can be resolved on a single run was increased by the use of multicolor dye labels. Primer sets labeled with dyes that can be distinguished by their emission wavelength generate products that are resolved according to fluorescence color as well as size (Fig. 11-11). Test DNA amplicons, allelic ladders, and size standards for multiple loci are thus run simultaneously through each capillary. Genotyping software such as GeneMapper (Applied Biosystems), STaR Call, and FMBIO Analysis Software (Hitachi Software Engineering) provide automated resolution of fluorescent dye colors and genotyping by comparison with the size standards and the allelic ladder.

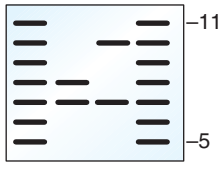
As in RFLP testing, an STR "match" is made by comparing profiles followed by probability calculations. The AmpliType HLA DQa Forensic DNA Amplification and Typing Kit (Promega) has been used in conjunction with the PM system to generate highly discriminatory allele frequencies. For example, the chance of a set of alleles occurring in two unrelated individuals at random is 1 in $10^6 - 7 \times 10^8$ Caucasians or 1 in $3 \times 10^6 - 3 \times 10^8$ African Americans.

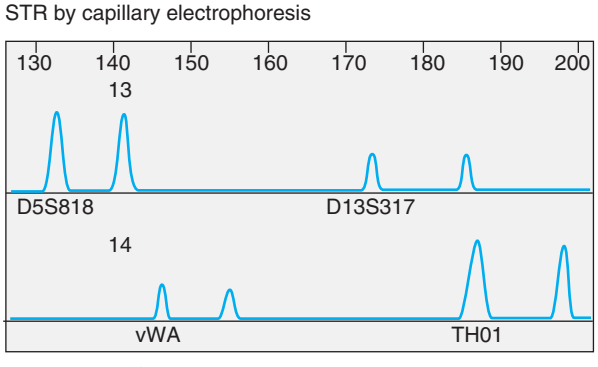
STR Nomenclature

The International Society for Forensic Genetics recommended nomenclature for STR loci in 1997.[8] STRs within genes are designated according to the gene name;



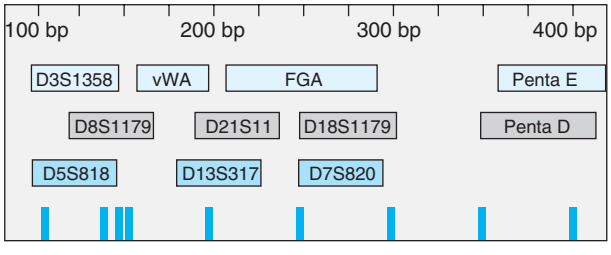
■ Figure 11-9 Multiple short tandem repeats can be resolved on a single gel. Here, four and five different loci are shown on the left and right gels, respectively. The allelic ladders show that the ranges of potential amplicon sizes do not overlap, allowing resolution of multiple loci in the same lane. Two individual genotypes are shown on the two gels.





■ Figure 11-10 STR analysis by capillary gel electrophoresis. Instead of bands on a gel (top), peaks of fluorescence on an electropherogram reveal the PCR product sizes (bottom). Alleles are determined by comparison with molecular weight markers and allelic ladders run through the capillary simultaneously with the sample amplicons.

for example, TH01 is in intron 1 of the human tyrosine hydroxylase gene on chromosome 11, and TPOX is in intron 10 of the human thyroid peroxidase gene on chromosome 2. These STRs do not have any phenotypic effect with respect to these genes. Non–gene associated STRs are designated by the D#S# system. D stands for DNA, the following number designates the chromosome



■ Figure 11-11 An illustration of the ranges of allele peak locations for selected STRs. By labeling primers with different fluorescent dye colors (FAM, JOE, and NED), STRs with overlapping size ranges can be resolved by color. The molecular weight markers (bottom) are labeled with the fluorescent dye ROX.

Table 11.2 **STR Locus Information*[50]**

STR	Locus	Chromosome	Repeat Sequence	Alleles†
CD4	Locus between CD4 and triosephosphate isomerase	12p	AAAAG‡	4, 6, 7, 8, 8′, 9, 10, 11, 12, 13, 14, 15
CSF1PO	c-fms protooncogene for CSF-1 receptor	5q	TAGA	6, 7, 8, 9, 10, 11, 12, 13, 14, 15
D3S1358		3p	TCTA§	8, 9, 10, 11, 12, 13, 14, 15, 15′, 15.2, 16, 16′, 16.2, 17, 17′, 17.1, 18, 18.3, 19, 20
D5S818		5q	AGAT	7, 8, 9, 10, 11, 12, 13, 14
D7S829		7q	GATA	7, 8, 9, 10, 11, 12, 13, 14, 15
D8S1179	Sequence tagged site	8q	TCTA	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
D13S317		13q	TATC	7, 8, 9, 10, 11, 12, 13, 14, 15
D16S539		16q	GATA	5, 8, 9, 10, 11, 12, 13, 14, 15
D18S51	Sequenced tagged site	18q	GAAA	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27
D21S11‖		21q	TCTG	24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38
F13A01	Coagulation factor IX	6p	GAAA	3.2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16
F13B	Factor XIII b	1q	TTTA	6, 7, 8, 9, 10, 11, 12
FESFPS	c-fes/fps protooncogene	15q	ATTT	7, 8, 9, 10, 11, 12, 13, 14
HPRTB	Hypoxanthine phosphoribosyl-transferase	Xq	TCTA	6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17
LPL	Lipoprotein lipase	8p	TTTA	7, 8, 9, 10, 11, 12, 13, 14
TH01	Tyrosine hydroxylase	11p	TCAT	5, 6, 7, 8, 9, 9.3, 10, 11
TPOX	Thyroid peroxidase	2p	TGAA	6, 7, 8, 9, 10, 11, 12, 13
vWA	Von Willebrand's factor	12p	TCTA	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21
PentaD		21q	AAAGA	2.2, 3.2, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17
PentaE		15q	AAAGA	5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 20.3, 21, 22, 23, 24

*http://www.cstl.nist.gov/div831/strbase/index.htm
†Some alleles have units with 1, 2, or 3 missing bases
‡In an alternate 8-repeat allele, one repeat sequence is AAAGG
§In alternate 15, 16, or 17 repeat alleles, one repeat sequence is TCTG
‖D21S11 has multiple alternate alleles

where the STR is located (1-22, X or Y). S refers to a unique segment, followed by a number registered in the International Genome Database (GDB). See Table 11.2 for some examples.

STRs are present all over the genome. Some of the STR loci commonly used for laboratory investigation are shown in Table 11.2. A comprehensive collection of STR information is available at cstl.nist.gov/biotech/strbase

Gender Identification

The **amelogenin locus** is a very useful marker often analyzed along with STR. The amelogenin gene, which is not an STR, is located on the X and Y chromosomes and is required for embryonic development and tooth maturation. The polymorphism is located in the second intron of the amelogenin gene. The Y allele of the gene is six base pairs larger in this region than the X allele. Amplification

Advanced Concepts

The GDB is overseen by the Human Genome Nomenclature Committee, a part of the Human Genome Organization (HUGO) located at University College, London. HUGO was established in 1989 as an international association of scientists involved in human genetics. The goal of HUGO is to promote and sustain international collaboration in the field of human genetics. The GDB was originally used to organize mapping data during the earliest days of the Human Genome Project (see Chapter 10). With the release of the human genome sequences and the development of polymerase chain reaction (PCR), the number of laboratories doing genetic testing has grown a thousand-fold. The GDB is still widely used as a source of information about PCR primers, PCR products, polymorphisms, and genetic testing. The use of information from GDB is unrestricted and available at http:www.gdb.org

Advanced Concepts

In 1997 the Federal Bureau of Investigation adopted 13 "core" loci as the Combined DNA Indexing System (**CODIS**). The loci are TPOX on chromosome 2, D3S1358 on chromosome 3, FGA on chromosome 4, D5S818 and CSF1PO on chromosome 5, D7S820 on chromosome 7, D8S1179 on chromosome 8, TH01 on chromosome 11, vWA on chromosome 12, D13S317 on chromosome 13, D16S539 on chromosome 16, D18S51 on chromosome 18, D21S11 on chromosome 2, and the amelogenin locus on the X and Y chromosome. The National Institute of Standards and Technology supplies Standard Reference Material for quality assurance of testing laboratories. The SRM certifies values for 22 STR loci, including CODIS and markers used by European forensic laboratories. Profiler Plus (Applied Biosystems) and PowerPlex (Promega) primer mixes include the CODIS loci.

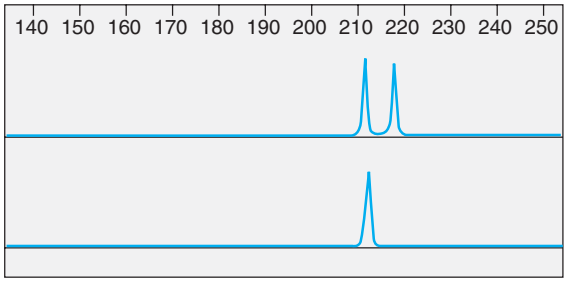


Figure 11-12 Males are heterozygous for the amelogenin locus (XY), and females are homozygous for this locus (XX). Amplification of amelogenin will produce a male-specific 218 bp product (Y allele) in addition to the 212 bp product found on the X chromosome (X allele).

and electrophoretic resolution reveals two bands or peaks for males (XY) and one band or peak for females (XX, Fig. 11-12). Some commercially available sets will contain primers to amplify the amelogenin polymorphism in addition to containing the STR primer sets.

Analysis of Test Results

Analysis of polymorphisms at multiple loci results in very high levels of discrimination (Table 11.3). Discovery of the same set of alleles from different sources or shared alleles between allegedly related individuals can be very strong evidence of identity, paternity, or relatedness. Results from such studies, however, must be expressed in terms of the background probability of chance matches.

DNA testing results in peak or band patterns that must be converted to genotype (allele identification) for com-

Table 11.3 **Matching Probability of STR Genotypes in Different Subpopulations**

	African American	White American	Hispanic American
8 loci	1/274,000,000	1/114,000,000	1/145,000,000
†9 loci	$1/5.18 \times 10^9$	$1/1.03 \times 10^9$	$1/1.84 \times 10^9$
*10 loci	$1/6.76 \times 10^{10}$	$1/9.61 \times 10^{10}$	
†12 loci	$1/4.61 \times 10^{12}$	$1/1.78 \times 10^{11}$	$1/4.75 \times 10^{11}$
†14 loci	$1/6.11 \times 10^{17}$	$1/9.96 \times 10^{17}$	$1/1.31 \times 10^{17}$
†16 loci	$1/7.64 \times 10^{17}$	$1/9.96 \times 10^{17}$	$1/1.31 \times 10^{17}$

*AmpliSTR Identifiler Kit (Applied Biosystems)
†PowerPlex Systems (Promega)

parison of results between laboratories. As described above, an STR **locus genotype** is defined by the number of repeats in the alleles. For instance, if the locus genotype in Figure 11-8 represented homologous chromosomes from an individual, the locus would be heterozygous, with 7 repeats on one chromosome and 8 repeats on the other. This locus would thus be designated 7/8 or 7,8. A homozygous locus (where both homologous chromosomes carry the same allele) is designated by the single number of repeats of that allele; for instance, 7/7 or 7,7. Some reports use a single number, such as 6 or 7 to designate a homozygous locus. **Microvariant** alleles containing partial repeat units are indicated by the number of complete repeats followed by a decimal point and then the number of bases in the partial repeat. For example, the 9.3 allele of the TH01 locus has 9 full 4-base pair repeat units and one repeat unit with 3 base pairs. Microvariants are detected as bands or peaks very close to the full-length allele (Fig. 11-13).

The genotype, or **profile**, of a specimen is the collection of alleles in all the locus genotypes tested. To determine the extent of certainty that one profile matches another, the occurrence of the detected genotype in the general or a defined population must be assessed.

A matching genotype is not necessarily an absolute determination of identity of an individual. **Genetic concordance** is a term used to express the situation where all locus genotypes (alleles) from two sources are the same. Concordance is interpreted as **inclusion** of a single individual as the donor of both genotypes. Two samples are considered different if at least one locus genotype differs (**exclusion**). An exception is paternity testing, in which mutational events may generate a new allele in the offspring, and this difference may not rule out paternity.

Matching requires clear and unambiguous laboratory results. As alleles are identified by gel resolution, good intragel precision (comparing bands or peaks on the same gel or capillary) and intergel precision (comparing bands or peaks of separate gels or capillaries) are important. In general, intergel precision is less stringent than intragel precision. This is not unexpected because the same samples may run with slightly different migration speeds on different gels. Some microvariant alleles differ by only a single base pair (see Fig. 11-13), so precision must be less than ±0.5 bp. Larger alleles, however, may show larger variation. The TH01 9.3 allele described above is an example. This allele must be distinguished from the 10 allele, which is a single base pair larger than the 9.3 allele.

Advanced Concepts

Alec Jeffreys' DNA profiling was the basis for the National DNA Database (NDNAD) launched in Britain in 1995. Under British law, the DNA profile of anyone convicted of a serious crime is stored on a database. The database now has DNA information on more than 250,000 people.

Created by the DNA Identification Act of 1994, the National DNA Index System (NDIS) is the federal level of the Combined DNA Indexing System (**CODIS**) used in the United States. There are three levels of CODIS: the Local DNA Index System (LDIS), State DNA Index System (SDIS), and NDIS. At the local level, CODIS software maintained by the Federal Bureau of Investigation (FBI) is used at the bench in sizing alleles. This information may be applied locally and/or submitted to the SDIS. At the state level, interlaboratory searching occurs. The state data may be sent to the NDIS. The SDIS and NDIS must adhere to the quality assurance standards recommended by the FBI. The original entries to these databases were RFLP profiles; all future entries will be STR profiles. As of 2005, there were 108,976 forensic DNA profiles and 2,390,740 convicted offender profiles in NDIS.

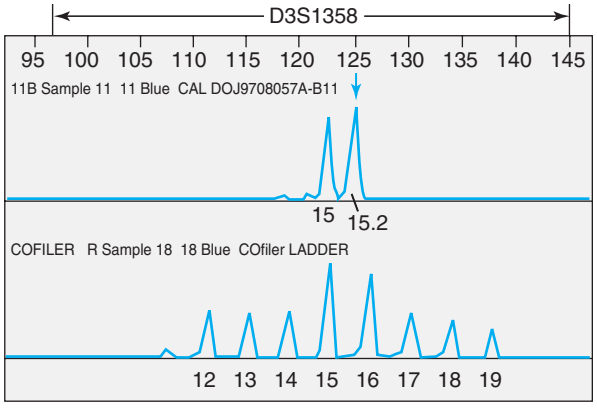


Figure 11-13 A microvariant allele (15.2) migrates between the full-length alleles. It is detected as a peak or band located very close to the full-length peak in an electropherogram.

Advanced Concepts

Artifacts such as air bubbles, crystals, and dye blobs, as well as sample contaminants, temperature variations, and voltage spikes, can interfere with consistent band migration. In addition, amplification artifacts occur during PCR. Some polymerases add an additional nontemplate adenine residue to the 3′ end of the PCR product. If this 3′ nucleotide addition does not include all the amplified products, a mixed set of amplicons will result in extra bands or peaks located very close together. **Stutter** is another anomaly of PCR amplification, in which the polymerase may miss a repeat during the replication process, resulting in two or more different species in the amplified product. These also appear as extra bands or peaks. Generally, the larger the repeat unit length, the less stutter is observed. These or other aberrant band patterns confuse the analysis software and can result in miscalling of alleles.

To establish identity of peaks from capillary electrophoresis (or peaks from densitometry tracings of gel data), the peak is assigned a position relative to some landmark within the gel lane or capillary, such as the loading well or the start of migration. Upon replicate resolutions of a band or peak, electrophoretic variations from capillary to capillary, lane to lane, or gel to gel may occur. Normalization of migration is achieved by relation of the migration of the test peaks to the simultaneous migration of size standards. Size standards can be internal (in the same gel lane or capillary) or external (in a separate gel lane). Even with normalization, however, tiny variations in position, height, and area of peaks or gel bands may persist. If the same fragments are run repeatedly, a distribution of observed sizes can be established. An acceptable range of sizes in this distribution is a **bin**. A bin can be thought of as an uncertainty window surrounding the mean position of each peak or band. All bands or peaks, therefore, that fall within this window are considered identical. Collection of all peaks or bands within a characteristic distribution of positions and areas is called **binning.**[9,10] Bins for each allele can be established manually in the laboratory. Alternatively, commercially available software has been designed to automatically bin and identify alleles.

Advanced Concepts

Binning can be performed in different ways using replicate peak height and position. To calculate the probability that two peaks are representative of the same allele, the proportion of alleles that fall within the uncertainty window (bin) must be determined. This proportion is represented exactly in **fixed bins** and approximated in **floating bins**. The fixed bin approach is an approximation of the more conservative floating bin approach.[9,56] An alternative assessment of allele certainty is the use of **locus-specific brackets.** In this approach, artificial "alleles" are designed to run at the high and low limit of the expected allele size. Identical alleles are expected to fall within this defined bracket.[10]

All peaks that fall within a bin are interpreted as representative of the same allele of a locus. Each band or peak in a genotype is binned and identified according to its migration characteristics. The group of bands or peaks makes up the characteristic pattern or profile of the specimen.

The number of loci tested must be taken into consideration in genotyping analysis. The more loci analyzed, the higher the probability that the locus genotype positively identifies an individual (**match probability**; see Table 11.3). Degraded, compromised, or mixed samples will affect the match probability, as all loci may not yield clear, informative results. Criteria for interpretation of results and determination of a true allele are established by each laboratory. These criteria should be based on validation studies and results reported from other laboratories. Periodic external proficiency testing should be performed to confirm the accuracy of test performance.

Matching of Profiles

Results from the analysis of polymorphisms are used to determine the probability of identity or inheritance of genetic markers or to **match** a particular marker or marker pattern. To establish the identity of an individual by an allele of a locus, the chance that the same allele could arise in the population randomly must be taken into account.

Advanced Concepts

The certainty of a matching pattern increases with decreased frequency of alleles in the general population. Under defined conditions, the relative frequency of two alleles in a population remains constant. This is **Hardy Weinberg equilibrium**, or the Hardy Weinberg Law.[57] The population frequency of two alleles, p and q, can be expressed mathematically as:

$$p^2 + 2pq + q^2 = 1.0$$

This equilibrium assumes a large population with random mating and no immigration, emigration, mutation, or natural selection. Under these circumstances, if enough individuals are assessed, a close approximation of the true allele frequency in the population can be determined.

The frequency of a set of alleles or a genotype in a population is the product of the frequency of each allele separately (the **product rule**). The product rule can be applied because of **linkage equilibrium**. Linkage equilibrium assumes that the loci are not associated with one another (genetically linked) in the genome. The overall frequency (OF) of a locus genotype consisting of n loci can be calculated as:

$$OF = F_1 \times F_2 \times F_3 \times \ldots F_n$$

where $F_{1\ldots n}$ represents the frequency of each individual allele in the population. Individual allele frequencies are determined by data collected from testing many individuals in general and defined populations. For example, at locus penta D on chromosome 21, the 5 allele has been previously determined to occur in 1 of 10 people in a theoretical population. At locus D7S829 on chromosome 7, the 8 allele has been previously observed in 1of 50 people in the same population. The overall frequency of the profile containing the loci penta D 5 allele and D7S829 8 allele would be $1/10 \times 1/50 = 1/500$. That is, that genotype or profile would be expected to occur in 1 out of every 500 randomly chosen members of that population. As should be apparent, the more loci tested, the greater the certainty that the profile is unique to a single individual in that population; that is, the overall frequency of the profile is very low. The overall frequencies in Table 11-3 illustrate this point.

Allele frequencies differ between **subpopulations** or ethnic groups. Different allele frequencies in subpopulations are determined through study of each ethnic group.[11] As can be seen from the data in Table 11.3, there are differences in the polymorphic nature of alleles in different subpopulations.

When identification using genotype profiles requires comparing the genotype of an unknown specimen with a known reference sample, for example, the genotype of evidence from a crime and the genotype of an individual from a database, the determination that the two genotypes match (are from the same person) is expressed in terms of a **likelihood ratio**. The likelihood ratio is the comparison of the probability that the two genotypes came from the same person with the probability that the two genotypes came from different persons, taking into account allele frequencies and linkage equilibrium in the population. A likelihood ratio greater than 1 is an indication that the probability is more likely, whereas a likelihood ratio of less than 1 indicates that the probability is less likely. If a likelihood ratio is 1000, the tested genotypes are 1000 times more likely to have come from the same person than from two randomly chosen members of the population. Or, in a random sampling of 100,000 members of a population, 100 people (100,000/1000) with the same genotype might be found. A simplified illustration can be made from the penta D and D7S829 example above. Suppose the penta D 5 and D7S829 8 profile was discovered in a specimen from an independent source. The likelihood that the profile came from the tested individual is 1, having been directly determined. The likelihood that the same profile could come from someone else in the population is 1/500. The likelihood ratio is 1/(1/500), or 500. The specimen material is 500 times more likely to have come from the tested individual than from some other person in the population.

When comparing genotypes with those in a database looking for a match, it is important to consider whether the database is representative of a population or subpopulation. It is also important to consider whether the population is homogeneous (a random mixture) with respect to the alleles tested.

Allelic Frequencies in Paternity Testing

A paternity test is designed to choose between two hypotheses: the test subject is not the father of the tested child (H_0), or the test subject is the father of the tested child (H_1). Paternity is first assessed by observation of

Advanced Concepts

Thomas Bayes proposed a theory to predict the chance of a future event based on the observation of the frequency of that event in the past. **Bayes' theorem** was found among his papers in an article published by The Royal Society in 1763 entitled "An Essay Towards Solving a Problem in the Doctrine of Chances" by the Reverend Thomas Bayes (Philosophical Transactions of the Royal Society, volume 53, pp. 370-418, 1763). The article had been published posthumously. In it, Bayes developed his theorem about conditional probability:

$$P(A|B) = \frac{P(A) \times P(B|A)}{P(B)}$$

That is, the probability that A will occur, given that B has occurred (posterior odds), is equal to the probability that B has occurred given that A has occurred (prior odds) times the quotient of the separate probabilities of A and B (likelihood ratio). Bayes' theorem is used in paternity testing and genetic association studies.[58]

shared alleles between the alleged father and the child (Fig. 11-14). Identity of shared alleles is a process of matching, as described above for identity testing.

A **paternity index**, or likelihood ratio of paternity, is calculated for each locus in which the alleged father and the child share an allele. The paternity index is an expression of how many times more likely the child's allele is inherited from the alleged father than by random occurrence of the allele in the general population. An allele that occurs frequently in the population has a low paternity index. A rare allele has a high paternity index. Table 11.4 shows the paternity index for each of four loci. The FESFPS 13 allele is rarer than the D16S539 9 allele. In this example, the child is 5.719 times more likely to have inherited the 9 allele of locus D16S539 from the alleged father than from another random man in the population. Similarly, the child is 15.41 times more likely to have inherited the 13 allele of FESFPS from the alleged father than by random occurrence. When each tested locus is on a different chromosome (not linked), the inheritance or occurrence of each allele can be considered an independent event. The paternity index for each locus, therefore,

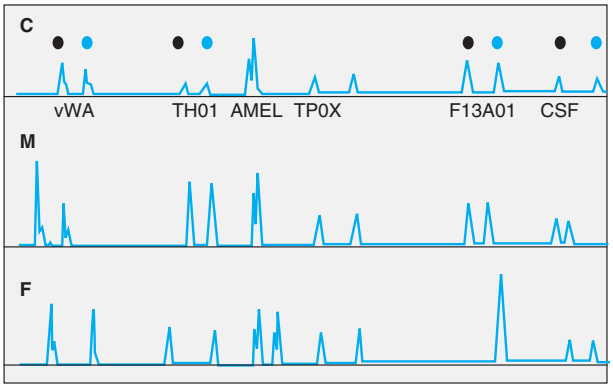


Figure 11-14 Electropherogram showing results from five STR loci and the amelogenin locus for a child (C), mother (M), and father (F). Note how the child has inherited one of each allele from the mother (black dots) and one from the father (green dots).

can be multiplied together to calculate the **combined paternity index** (CPI), which summarizes and evaluates the genotype information. The CPI for the data shown in Table 11-4 is:

$$CPI = 5.719 \times 8.932 \times 15.41 \times 10.22 = 8{,}044.931$$

This indicates that the child is 8045 times more likely to have inherited the four observed alleles from the alleged father than from another man in the population.

If a paternal allele does not match between the alleged father and the child, H_1 for that allele is 0. One might assume, therefore, that the nonmatching allele paternity index of 0 would make the CPI 0. This is not the case. Nonmatching alleles between the alleged father and the child found at one locus (exclusion) is traditionally not regarded as a demonstration of nonpaternity because of the possibility of mutation. Although mutations were quite rare in the traditional RFLP systems, analysis of 12 or more STR loci may occasionally reveal one or two

Table 11.4 **Example Data From a Paternity Test Showing Inclusion**

Allele	Child	Alleged Father	Shared Allele	Paternity Index
D16S539	8, 9	9, 10	9	5.719
D5S818	10, 12	7, 12	12	8.932
FESFPS	9, 13	13, 14	13	15.41
F13A01	4, 5	5, 7	5	10.22

mutations resulting in nonmatching alleles even if the man is the father. To account for mutations, the paternity index for nonmatching alleles is calculated as:

$$\text{paternity index for a mutant allele} = \mu$$

where μ is the observed mutation rate (mutations/meiosis) of the locus. The American Association of Blood Banks has collected data on mutation rates in STR loci (Table 11.5).

Using these data, in the case of a nonmatching allele, H_1 is not 0 but μ.

In a paternity report, the combined paternity index is accompanied by the **probability of paternity**, a number calculated from the paternity index (genetic evidence) and prior odds (nongenetic evidence). For the prior odds, the laboratory as a neutral party assumes a 50/50 chance that the test subject is the father. The probability of paternity is, therefore:

$$\frac{\text{CPI} \times \text{prior odds}}{(\text{CPI} \times \text{prior odds}) + (1 - \text{prior odds})}$$

$$= \frac{\text{CPI} \times 0.50}{(\text{CPI} \times 0.50) + (1 - 0.50)}$$

Table 11.5 **Observed Mutation Rates in Paternity Tests Using STR Loci**

STR Locus	Mutation Rate (%)
D1S1338	0.09
D3S1358	0.13
D5S818	0.12
D7S820	0.10
D8S1179	0.13
D13S317	0.15
D16S539	0.11
D18S51	0.25
D19S433	0.11
D21S11	0.21
CSF1PO	0.16
FGA	0.30
TH01	0.01
TPOX	0.01
VWA	0.16
F13A01	0.05
FESFPS	0.05
F13B	0.03
LPL	0.05
Penta D	0.13
Penta E	0.16

Advanced Concepts

Based on studies showing that the majority of STR mutations are gains or losses of a single repeat,[20] Brenner proposed that the paternity index for a mutant allele must take into account the nature of the mutation; that is, loss or gain of one or more repeats.[59] The loss of one repeat is much more likely in a single mutation event than the loss of two or more repeats. According to Brenner's formula:

Paternity index for a mutant allele = $\mu/(4\ P(Q))$ (for a single repeat difference)

Paternity index for a mutant allele = $\mu/(40\ P(Q))$ (for a two-repeat difference) and so forth.

P(Q) is the frequency or probability of occurrence of the normal allele, Q, in the population.

In the example illustrated previously, the CPI is 8,044.931. The probability of paternity is:

$$\frac{8044.931 \times 0.50}{(8044.931 \times 0.50) + 0.50} = 0.999987$$

The genetic evidence (CPI) has changed the probability of paternity (prior odds) of 50% to 99.9987%.

There is some disagreement about the assumption of 50% prior odds. Using different prior odds assumptions changes the final probability of paternity (Table 11.6). As can be observed from the table, however, at CPI over 100 the differences become less significant.

Sibling Tests

Polymorphisms are also used to generate a probability of siblings or other blood relationships.[12] A sibling test is a more complicated statistical analysis than a paternity

Table 11.6 **Odds of Paternity Using Different Prior Odds Assumptions**

	Prior Odds				
CPI	10%	25%	50%	75%	90%
5	0.36	0.63	0.83	0.94	0.98
9	0.50	0.75	0.90	0.96	0.98
19	0.68	0.86	0.95	0.98	0.994
99	0.92	0.97	0.99	0.997	0.999
999	0.99	0.997	0.999	0.9997	0.9999

test.[13,14] **A full sibling test** is a determination of the likelihood that two people tested share a common mother and father. **A half sibling test** is a determination of the likelihood that two people tested share a common parent (mother or father). The likelihood ratio generated by a sibling test is sometimes called a **kinship index, sibling index**, or **combined sibling index**.

A test to determine the possibility of an aunt or uncle relationship, also known as **avuncular testing**, measures the probabilities that two alleged relatives are related as either an aunt or an uncle of a niece or nephew. The probability of relatedness is based on the number of shared alleles between the tested individuals. As with paternity and identity testing, allele frequency in the population will affect the significance of the final results. The probabilities can be increased greatly if other known relatives, such as a parent of the niece or nephew, are available for testing. Determination of first- and second-degree relationships is important for genetic studies because linkage mapping of disease genes in populations can be affected by undetected familial relationships.[15]

Y-STR

Unlike conventional STRs (**autosomal STRs**), where each locus is defined by two alleles, one from each parent, Y-STRs are represented only once per genome and only in males (Fig. 11-15). A set of Y-STR alleles comprises a **haplotype**, or series of linked alleles always inherited together, because the Y chromosome cannot exchange information (recombine) with another Y chromosome. Thus, marker alleles on the Y chromosome are inherited from generation to generation in a single block. This means that the frequency of entire Y-STR profiles (haplotypes) in a given population can be determined by empirical studies. For example, if a combination of alleles (haplotype) was observed only two times in a test of 200 unrelated males, that haplotype is expected to occur with a frequency of approximately 1 in 100 males tested in the future. The discrimination power of Y-haplotype testing will depend on the number of subjects tested and will always be less commanding than with autosomal STR.

Despite being a less powerful system for identification, STR polymorphisms on the Y chromosome have unique characteristics that have been exploited for forensic, lineage, and population studies as well as kinship testing.[16] Except for rare mutation events, every male member of a family (brothers, uncles, cousins, and grandparents) will have the same Y-chromosome haplotype. Thus Y-chromosome inheritance can be applied to lineage, population, and human migration studies.

As all male relatives in a family will share the same allele combination or profile, the statistical significance of a Y-STR DNA match cannot be assessed by multiplying likelihood ratios as was described above for autosomal STR. Instead of allele frequency used in the match calculations of STR, haplotype frequencies are used. Estimation of haplotype frequencies, however, is limited by the number of known Y haplotypes. This smaller data set accounts for the reduced inclusion probabilities and a discrimination rate that is significantly lower than that for autosomal STR polymorphisms. Traditional STR loci are, therefore, preferred for identity or relationship analyses, and the Y-STRs are used to aid in special situations; for instance, in confirming sibship between males who share commonly occurring alleles, that is, have a low likelihood ratio based on traditional STRs.

Y-STRs have been utilized in forensic tests where evidence consists of a mixture of male and female DNA, such as semen, saliva, other body secretions or fingernail scrapings. For instance in specimens from evidence of rape, the female DNA may be in vast excess (more than 100-fold) compared to the male DNA in the sample.[17] Autosomal STR are not consistently informative under these circumstances. Using Y-specific primers, however, Y-STR can be specifically amplified from the male-female mixture resulting in an analyzable marker that has no female background. This affords a more accurate identification of the male donor.

Y haplotyping is also used in lineage studies involving paternally linked relationships and identification. The Y-STR/paternal lineage test can determine whether two or more males have a common paternal ancestor. In addition to family history studies, the results of a paternal lineage test serve as supportive evidence for adoptees and their biological relatives or for individuals making inheritance and Social Security benefit claims.

As Y chromosomes are inherited intact, spontaneous mutations in the DNA sequence of the Y chromosome can be used to follow human migration patterns and historical lineages. Y-chromosome genotyping has been used in studies designed to locate the geographical origin of all human beings.[18]

The Y chromosome has a low mutation rate. The overall mutation rate for Y chromosome loci is estimated at 1.72–4.27 per thousand alleles.[19,20] Assuming that Y

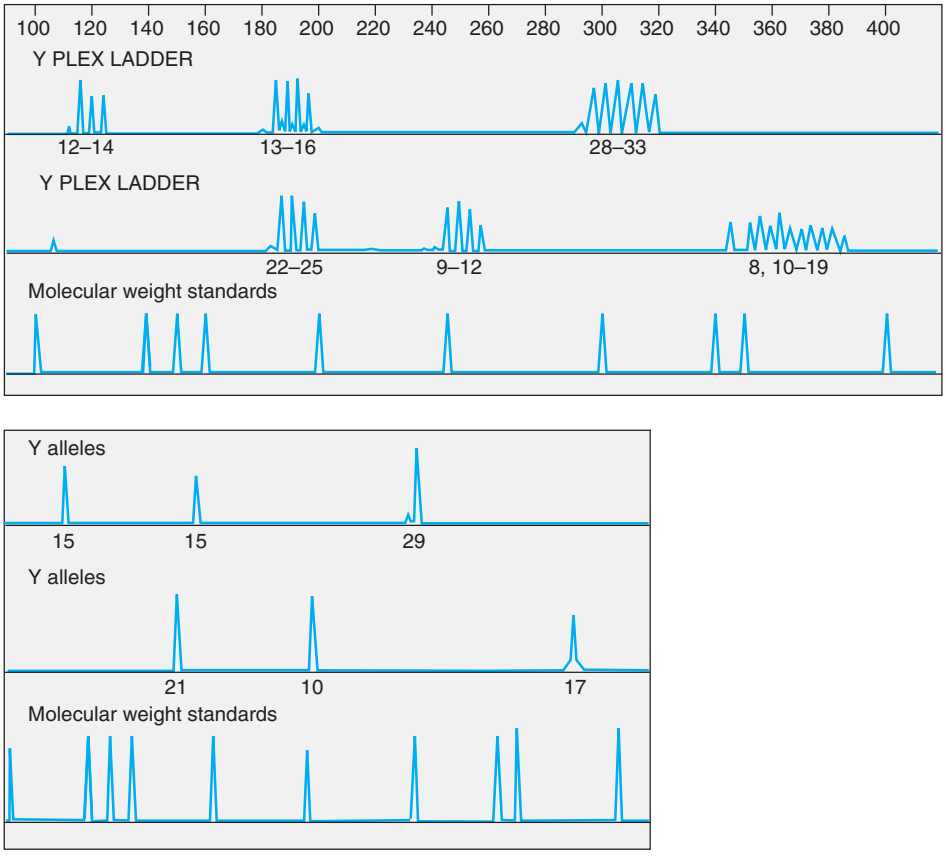


Figure 11-15 Electropherogram showing allelic ladders for six STR loci in the Y-Plex 6 system (top panel) and a single haplotype (bottom panel). Molecular weight standards are shown at the bottom of each.

chromosome mutations generally occur once every 500 generations/locus,[21] for 25 loci, 1 locus should have a mutation every 20 generations (500 generations/25 markers = 20 generations). This low mutation rate makes it possible to investigate the paternal lineage over several generations. It is also useful for missing persons' cases in which reference samples can be obtained from paternally related males.

A list of informative Y-STRs is shown in Table 11.7. Several Y-STRs are located in regions that are duplicated on the Y chromosome. DSY389I and DSY389II are examples of a duplicated locus. A quadruplicated locus, DSY464, has also been reported.[22] Like autosomal STRs, Y-STRs have microvariant alleles containing incomplete repeats and alleles containing repeat sequence differences. Reagent systems consisting of multiplexed primers for identification of Y-STRs are available commercially; for example, the Powerplex Y System, which contains 12 Y loci (Promega); the AmpliSTR Y-filer, which contains 17 Y loci (Applied Biosystems); and the Y-Plex 6, which contains 6 Y loci (Reliagene).

Matching With Y-STRs

Matching probabilities from Y-STR data are determined differently than for the autosomal STR. **Haplotype diversity** (HD) can be calculated from the frequency of occurrence of a given haplotype in a tested population. The probability of two random males sharing the same haplotype is estimated at 1-HD. Another measure of profile uniqueness, the **discriminatory capacity** (DC), is determined by the number of different haplotypes

Advanced Concepts

The European Y chromosome typing community has established a set of Y-STR loci termed the **minimal haplotype** (see http://www.ystr.org). The minimal haplotype consists of Y-STR markers DYS19, DYS389I, DYS389II, DYS390I, DYS391, DYS392, DYS393, and DYS385.[60] An "**extended haplotype**" includes all of the loci from the minimal haplotype plus the highly polymorphic dinucleotide repeat YCAII.

seen in the tested population and the total number of samples in the population. DC expresses the percentage of males in a population who can be identified by a given haplotype. Just as the number of loci included in an autosomal STR genotype increases the power of discrimination, DC is increased by increasing the number of loci defining a haplotype. For instance, the loci tested in the Y-Plex 6 system can distinguish 82% of African-American males. Using 22 loci raises the DC to almost 99% (Table 11.8).

As there is no recombination between loci on the Y chromosome, the product rule cannot be applied. The

Table 11.7 **Y-STR Locus Information*[51–53]**

Y-STR	Repeat Sequence†	Alleles
DYS19	$[TAGA]_3TAGG[TAGA]_n$	10, 11, 12, 13, 14, 15, 16, 17, 18, 19
DYS385	$[GAAA]_n$	7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 16.3, 17, 17.2 17.3, 18, 19, 20, 21, 22, 23, 24, 28
DYS388	$[CAA]_n$	10, 11, 12, 13, 14, 15, 16, 17, 18
DYS389 I‡	$[TCTG]_q$ $[TCTA]_r$	9, 10, 11, 12, 13, 14, 15, 16, 17
DYS389 II‡	$[TCTG]_n[TCTA]_p[TCTG]_q[TCTA]_r$	26, 27, 28, 28', 29, 29', 29", 29"', 30, 30' 30", 30"'. 31, 31' 31", 32, 32,', 33, 34
DYS390	$[TCTG]_n[TCTA]_m[TCTG]_p[TCTA]$	17,18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28
DYS391	$[TCTA]_n$	6, 7, 8, 9, 10, 11, 12, 13, 14
DYS392	$[TAT]_n$	6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17
DYS393	$[AGAT]_n$	9, 10, 11, 12, 13, 14
DYS426	$[CAA]_n$	6.2, 9, 10, 11, 12, 13, 14
DYS434	$[CTAT]_n$	8, 9, 10, 11
DYS437	$[TCTA]_n[TCTG]_2[TCTA]_4$	13, 14, 15, 16, 17
DYS438	$[TTTTC]_n$	6, 7, 8, 9, 10, 11, 12, 13, 14
DYS439	$[GATA]_n$	9, 10, 11, 12, 13, 14
DYS439 (Y-GATA-A4)	$[GATA]_n$	9, 10, 11, 12, 13, 14
DYS441	$[CCTT]_n$	8, 10.1, 11, 11.1, 12, 13, 13.1, 14, 14.3, 15, 16, 17, 18, 19, 20
DYS442	$[TATC]_n$	8, 9, 10, 11, 12, 12.1, 13, 14, 15
DYS444	$[TAGA]_n$	9, 10, 11, 12, 13, 14, 15, 16
DYS445	$[TTTA]_n$	6, 7, 8, 9, 10, 10.1, 11, 12, 13, 14
DYS446	$[AGAGA]_n$	8, 9, 10, 11, 12, 13, 14, 15, 15.1, 16, 17, 18, 19, 19.1, 20, 21, 22, 23
DYS447	$[TTATA]_n$	15, 16, 17, 18, 19, 19.1, 20, 21, 22, 22.2, 22.4, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36
DYS448	$[AGAGAT]_n$	17, 19, 19.2, 20, 20.2, 20.4, 21, 21.2, 21.4, 22, 22.2, 23, 23.4, 24, 24.5, 25, 26, 27

Continued on following page

Table 11.7 **Y-STR Locus Information*[51–53]** *(continued)*

Y-STR	Repeat Sequence†	Alleles
DYS449	$[GAAA]_n$	23, 23.4, 24, 24.5, 25, 26, 27, 27.2, 28, 28.2, 29, 29.2, 30, 30.2, 31, 32, 32.2, 33, 33.2, 34, 35, 36, 37, 37.3, 38
DYS452	$[TATAC]_n$	24, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35
DYS454	$[TTAT]_n$	6, 7, 8, 9, 10, 11, 12, 13
DYS455	$[TTAT]_n$	7, 8, 9, 10, 11, 12, 13
DYS456	$[AGAT]_n$	11, 12, 13, 14, 15, 16, 17, 18, 19, 20
DYS458	$[CTTT]_n$	12, 12.2, 13, 14, 15, 15.2, 16, 16.1, 16.2, 17, 17.2, 18, 18.2, 19, 19.2, 20, 20.2, 21
DYS460 (Y-GATA-A7.1)	$[ATAG]_n$	7, 8, 9, 10, 10.1, 11, 12, 13

*http://www.cstl.nist.gov/div831/strbase/index.htm
†Some alleles contain repeats with 1, 2, or 3 bases missing
‡DYS389 I and II is a duplicated locus

results of a Y typing can be reported accompanied by the number of observations or frequency of the analyzed haplotype in a database of adequate size. Suppose a haplotype containing the 17 allele of DYS390 occurs in only 23% of men in a database of 12,400. However, if that same haplotype contains the 21 allele of DYS446, only 6% of the men will have haplotypes containing the DYS390 17 and DYS446 21 alleles. If the 11 allele of DYS455 and the 15 allele of DYS458 are also present, only 1 out of 12,400 men in the population has a haplotype containing all four alleles. The uniqueness of this haplotype is strong evidence that a match is not the result of a random coincidence, which gives extra support to the hypothesis that an independent source with this haplotype comes from an individual or a paternal relative. Even with a 99.9% DC, however, the matching probability is orders of magnitude lower than that for autosomal STR.

Table 11.8 **Discriminatory Capacity of Y-STR Genotypes in Different Subpopulations[54]**

	African American (%)	White American (%)	Hispanic American (%)
*6 loci	82.3	68.9	78.3
†9 loci	84.6	74.8	85.1
‡11 loci	91.3	83.8	90.3
§17 loci	99.1	98.8	98.3
‖20 loci	98.5	97.2	98.6
#22 loci	98.9	99.6	99.3

*Y-Plex 6 (DYS19, DYS390, DYS391, DYS393, DYS389II, and DYS385)
†European minimal haplotype
‡Minimal haplotype + SWGDAM
§AmpliSTR Y-filer as reported by Applied Biosystems
‖Y-STR 20 plex (Minimal haplotype plus DYS388, DYS426, DYS437, DYS439, DYS460, H4, DYS438 DYS447 and DYS448)
#Y-STR 22 plex

Y-chromosome haplotypes can be used to exclude paternity. Taking into account the mutation rate of each allele, any alleles that differ between the male child and the alleged father are strong evidence for nonpaternity. Conversely, if a Y haplotype is shared between a child and alleged father, a paternity index can be calculated in a manner similar to that of the autosomal STR analysis. For example, suppose 6 Y-STR alleles are tested and match between the alleged father and child. If the haplotype has not been observed before in the population, the occurrence of that haplotype in the population database is 0/1200, and the haplotype frequency will be 1/1200, or 0.0008333. The paternity index (PI) is the probability that a man with that haplotype could produce one sperm carrying the haplotype (H_0), divided by the probability that a random man could produce one sperm carrying the haplotype (H_1). The PI is then $1/0.0008333 = 1200$. With a prior probability of 0.5, the probability of paternity is $(1200 \times 0.5)/[(1200 \times 0.5) + 0.5]$ or 99.9%. This result, however, does not exclude patrilineal relatives of the alleged father.

Y-STRs also provide marker loci for Y-chromosome, or **surname**, tests to determine ancestry. For example, a group of males of a strictly male descent line (having the same last name or surname) is expected to be related to a common male ancestor. Therefore, they should all share

the same Y-chromosome alleles (except for mutations, which should be minimal, given 1 mutation per 20 generations, as explained above). The Y-chromosome haplotype does not provide information about degree of relatedness, just inclusion or exclusion from the family. An analysis to find a most recent common ancestor (MRCA) is possible, however, using a combination of researched family histories, Y-STR test results, and statistical formulas for mutation frequencies.

Engraftment Testing Using DNA Polymorphisms

Bone marrow transplantation is a method used to treat malignant and nonmalignant blood disorders, as well as some solid tumors. The transplant approach can be **autologous** (from self), in which cells from the patient's own bone marrow are removed and stored. The patient then receives high doses of chemotherapy and/or radiotherapy. The portion of marrow previously removed from the patient may also be purged of cancer cells before being returned to the patient. Alternatively, **allogeneic** transplants (between two individuals) are used. The **donor** supplies healthy cells to the **recipient** patient (Fig. 11-16). Donor cells are supplied as bone marrow, peripheral blood stem cells (also called **hematopoetic stem cells**), or umbilical cord blood. To assure successful establishment of the transplanted donor cells, donor and recipient immune compatibility is tested prior to the transplant by HLA typing (see Chapter 15).

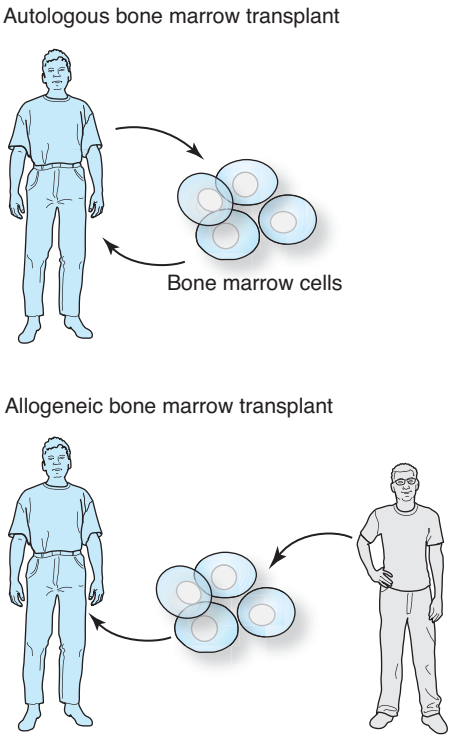


Figure 11-16 In autologous bone marrow transplant (top), bone marrow cells are taken from the patient, purged, and replaced in the patient after conditioning treatment. In allogeneic transplant (bottom), bone marrow cells are taken from another genetically compatible individual (donor) and given to the patient.

In **myeloablative** transplant strategies, high doses of therapy completely remove the recipient bone marrow, particularly the stem cells that give rise to all the other cells in the marrow (**conditioning**). The allogeneic or autologous stem cells are then expected to re-establish a new bone marrow in the recipient (**engraftment**). The toxicity of this procedure can be avoided by the use of **nonmyeloablative** transplant procedures or **minitransplants**. In this approach, pretransplant therapy will not completely remove the recipient bone marrow. The donor bone marrow is expected to eradicate the remaining recipient cells through recognition of residual recipient cells as foreign to the new bone marrow. This process also imparts a graft-versus-leukemia or **graft-versus-tumor** (GVT) effect, which is the same process as **graft-versus-host disease** (GVHD). The T-cell fraction of the donor marrow is particularly important for engraftment and for GVT effect. Efforts to avoid GVHD by removing the T-cell fraction before infusion of donor cells has resulted in increased incidence of graft failure and relapse.

The first phase of allogeneic transplantation is donor matching, in which potential donors are tested for immunological compatibility. This is performed by examining the human leukocyte antigen (HLA) locus using sequence-specific PCR or by sequence-based typing (see Chapter 15). Sequence polymorphisms (alleles) in the HLA locus are compared with those of the recipient to determine which donor would be most tolerated by the recipient immune system. Donors may be known or related to the patient or anonymous unrelated contributors (**matched unrelated donor**).

Stem cells may also be acquired from donated umbilical cord blood. After conditioning and infusion with the donor cells, the patient enters the engraftment phase, in which the donor cells reconstitute the recipient's bone marrow. Once a successful engraftment of donor cells is

established, the recipient is a genetic **chimera**; that is, the recipient has body and blood cells of separate genetic origins.

The engraftment of donor cells in the recipient must be monitored, especially in the first 90 days after the transplant. This requires a method that can distinguish donor cells from recipient cells. Earlier methods included red blood cell phenotyping, immunoglobulin allotyping, HLA typing, karyotyping, and fluorescence in situ hybridization analysis. Each of these methods has drawbacks. Some require months before engraftment can be detected. Others are labor-intensive or restricted to sex mismatched donor-recipient pairs.

DNA typing has become the method of choice for engraftment monitoring.[23,24] Because all individuals, except identical twins, have unique DNA polymorphisms, donor cells can be monitored by following donor polymorphisms in the recipient blood and bone marrow. Although RFLP can effectively distinguish donor and recipient cells, the detection of RFLP requires use of the Southern blot method, which is too labor-intensive and slow for this application. In comparison, small VNTRs and STRs are easily detected by PCR (see Fig. 11-9). PCR amplification of VNTRs and STRs is preferable because of the increased rapidity and the 0.5%–1% sensitivity achievable with PCR. Sensitivity can be raised to 0.01% using Y-STR, but this approach is lim-ited to those transplants from a female donor to a male recipient.[25,26]

In the laboratory, there are two parts to engraftment/chimerism DNA testing. Before the transplant, several polymorphic loci in the donor and recipient cells must be screened to find at least one **informative** locus; that is, one in which donor alleles differ from the recipient alleles. **Noninformative** loci are those in which the donor and the recipient have the same alleles. In **donor-informative** loci, donor and recipient share one allele, and the donor has a unique allele. Conversely, in **recipient-informative** loci, the unique allele is in the recipient (Fig. 11-17). The second part of the testing process is the engraftment analysis, which is performed at specified intervals after the transplant. In the engraftment analysis, the recipient blood and bone marrow are tested to determine the presence of donor cells using the informative and/or recipient informative loci.

Pretransplant analysis and engraftment were measured in early studies by amplification of small VNTRs and resolution of amplified fragments on polyacrylamide gels with silver stain detection.[27] Before the transplant, the screen for informative loci was based on band patterns of the PCR products, as illustrated in Figure 11-17. After the transplant, analysis of the gel band pattern from the blood and bone marrow of the recipient revealed one of three different states: **full chimerism**, in which only the donor alleles were detected in the recipient; **mixed chimerism,** in which a mixture of donor and recipient alleles was present, or **graft failure**, in which only recipient alleles were detectable (Fig. 11-18).

Currently, PCR amplification of STRs, resolution by capillary electrophoresis, and fluorescent detection is the preferred method. This procedure provides ease of use, accurate quantitation of the percentage of donor/recipient cells, and high sensitivity with minimal sample requirements.

Donor and recipient DNA for allele screening prior to transplant can be isolated from blood or buccal cells. One

Advanced Concepts

Chimerism is different from **mosaicism**. A chimera is an individual carrying two populations of cells that arose from different zygotes. In a mosaic, cells arising from the same zygote have undergone a genetic event, resulting in two clones of phenotypically different cells in the same individual.

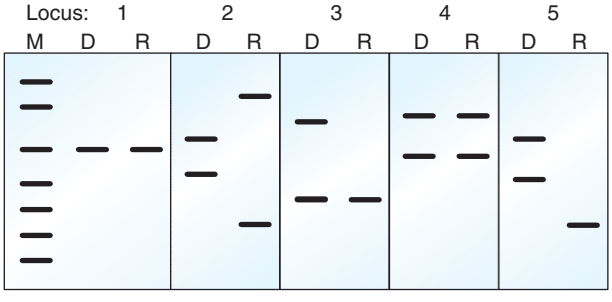


Figure 11-17 Band patterns of five different loci comparing donor (D) and recipient (R) alleles. The second and fifth loci are informative. The first and fourth loci are noninformative. The third locus is donor-informative.

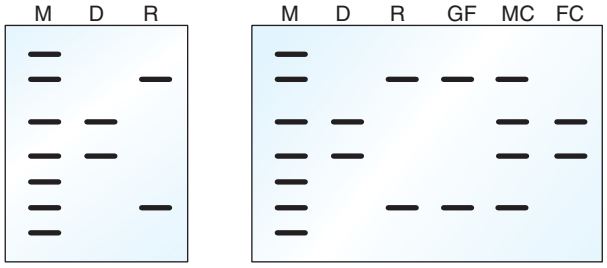


Figure 11-18 Band patterns after PAGE analysis of VNTR. First, before the transplant, several VNTR must be screened to find informative loci that differ in pattern between the donor and recipient. One such marker is shown at left (M = molecular weight marker, D = donor, R = recipient). After the transplant, the band patterns can be used to distinguish between graft failure (GF), mixed chimerism (MC), or full chimerism (FC).

ng of DNA is reportedly sufficient for screening of multiple loci; however, 10 ng is a more practical lower limit. Multiple loci can be screened simultaneously using multiplex PCR. Although not validated for engraftment testing, several systems designed for human identification, such as Promega's PowerPlex and Applied Biosystem's AmpliSTR Identifiler and Profiler, may be used for this purpose. The AmpliSTR Yfiler may also be useful for sex mismatched donor/recipient pairs. Figure 11-19 shows the five tetramethylrhodamine (TMR)–labeled loci from the PowerPlex system. A total of nine loci are amplified simultaneously by this set of multiplexed primers. Although multiplex primer systems are optimized for consistent results, all loci may not amplify with equal efficiency in a multiplex reaction. For example, the amelogenin locus in Figure 11-19 did not amplify as well as the other four loci in the multiplex. This is apparent from the lower peak heights in the amelogenin products compared with the products of the other primers.

Advanced Concepts

A more defined condition can be uncovered by cell type separation. Some cell fractions, such as granulocytes, engraft before others. Isolated granulocytes may show full chimerism while the T-cell fraction still shows mixed chimerism. This is a case of **split chimerism**.

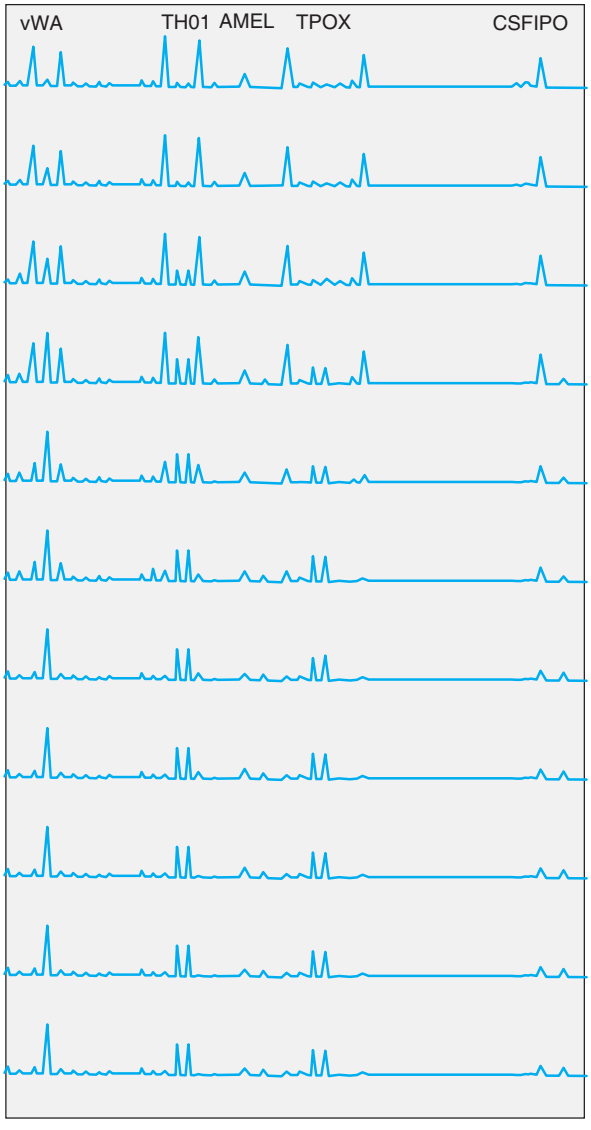


Figure 11-19 Multiplex PCR showing DNA mixtures from two unrelated individuals (top and bottom trace) showing peak patterns for vWA, TH01, Amelogenin, TPOX, and CSF1PO loci. The center traces are stepwise mixtures of the two genotypes.

Although the instrumentation used for this method is the same as that used for sequence analysis (see Chapter 10), investigating peak sizes and peak areas is distinguished from sequence analysis as **fragment analysis** and sometimes requires adjustment of the instrument or capil-

lary polymer. Automatic detection will generate an electropherogram as shown in Figure 11-20. Informative and noninformative loci will appear as nonmatching or matching donor and recipient peaks, respectively. Many combinations of donor/recipient peaks are possible. Optimal loci for analysis should be clean peaks without **stutter,** especially stutter peaks that co-migrate with informative peaks, nonspecific amplified peaks (misprimes), or other technical artifacts.[28] Ideally, the chosen locus should have at least one recipient informative allele. This is to assure direct detection of minimal amounts of residual recipient cells. If the recipient is male and the donor is female, the amelogenin locus supplies a recipient informative locus. Good separation (ideally, but not necessarily, by two repeat units) of the recipient and donor alleles is desirable for ease of discrimination in the post-transplant testing. The choices of informative alleles are more limited in related donor-recipient pairs, as they are likely to share alleles. Unrelated donor-recipient pairs, on the other hand, will yield more options.

After the transplant, the recipient is tested on a schedule determined by the clinician or according to consensus recommendations.[29] With modern nonmyeloablative or reduced-intensity pretransplant protocols, testing is recommended at 1, 3, 6, and 12 months. Because early patterns of engraftment may predict GVHD or graft failure after nonmyeloblative treatments, even more frequent blood testing may be necessary, such as 1, 2, and 3 months after transplant. Bone marrow specimens can most conveniently be taken at the time of bone marrow biopsy following the transplant, with blood specimens taken in intervening periods. Usually, 3–5 mL of bone marrow or 5 mL of blood is more than sufficient for analysis; however, specimens collected soon after the transplant may be hypocellular so that larger volumes (5–7 mL bone marrow, 10 mL blood) may be required.

Quantification of percent recipient and donor post-transplant is performed using the informative locus or loci selected during the pretransplant informative analysis. The raw data for these calculations are the areas under the peaks generated by the PCR products after amplification. The emission from the fluorescent dyes attached to the primers and thus to the ends of the PCR products is collected as each product migrates past the detector. The fluorescent signal is converted into fluorescence units by the

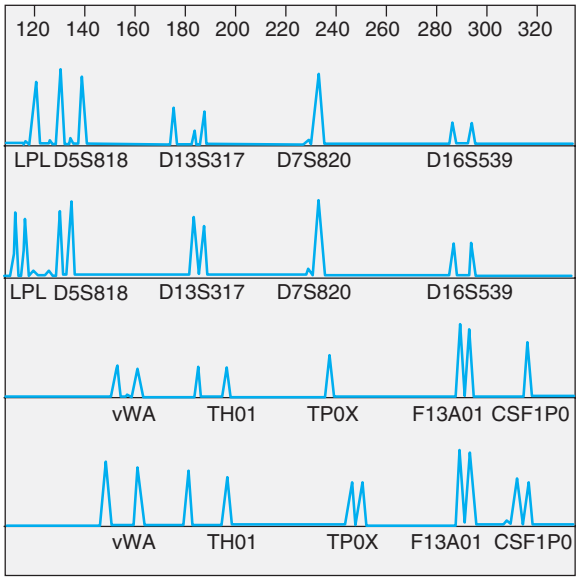


Figure 11-20 Screening of 10 loci for informative alleles. Recipient peak patterns (first and third scans) are compared with donor patterns (second and fourth scans). LPL, D5S818, D13S317, vWA, TH01, TP0X, and CSF1P0 are informative.

Advanced Concepts

Occasionally, specimens may be received in the laboratory after engraftment without pre-engraftment information. In this case, the blood or bone marrow of the recipient is not acceptable for determination of recipient-specific alleles because the alleles present are likely to represent both donor and recipient. The specimen can be processed using the amelogenin locus or Y-STR markers if the donor and recipient are of different sexes, preferably female donor and male recipient. Another option is to use an alternate source of recipient DNA such as buccal cells, skin biopsy sample, or stored specimens or DNA from previous testing. Because of the nature of lymphocyte migration, however, skin and buccal cells may also have donor alleles due to the presence of donor lymphocytes in these tissues. The best approach is to ensure informative analysis of the donor and recipient as part of the pretransplant schedule.

Advanced Concepts

Positive or negative selection techniques may be used to test specific cell lineages. For example, analysis of the T-cell fraction separately is used to monitor graft-versus-tumor activity. T cells may comprise 10% of peripheral blood leukocytes and 3% of bone marrow cells following allogeneic transplantation. Analysis of unfractionated blood and especially bone marrow where all other lineages are 100% may miss split chimerism in the T-cell fraction. T-lineage–specific chimerism will therefore increase the sensitivity of the engraftment analysis, particularly after nonmyeloablative and immunoablative pretransplant treatments.

T cells are conveniently separated from whole blood using magnetized polymer particles (beads), such as MicroBeads (MicroBeads AS), DynaBeads (Dynal), or EasySep (StemCell Technologies), attached to pan-T antibodies (anti-CD3). To isolate T cells, white blood cells isolated by density gradient centrifugation are mixed with the beads in saline or phosphate-buffered saline and incubated to allow the antibodies on the beads to bind to the CD3 antigens on the T-cell surface. With the beads–T cells immobilized by an external magnet, the supernatant containing non-T cells is decanted. After another saline wash, the T cells are collected and lysed for DNA isolation. It is not necessary to detach the T cells from the beads.

Automated cell sorter systems, such as the autoMACS separator (Miltenyi Biotec), may also be used for this purpose. With a positive selection program, the instrument is capable of isolating up to 2×10^8 pure T cells per separation. Unwanted cells can be removed with the depletion programs.

computer software. The software displays the PCR products as peaks of fluorescence units (y-axis) vs. migration speed (x-axis). The amount of fluorescence in each product or peak is represented as the area under the peak. This number is provided by the software and is used to calculate the percent recipient and donor (Fig. 11-21).

There are several formulas for percent calculations, depending on the configuration of the donor and recipient peaks. For homozygous or heterozygous donor and recipient peaks with no shared alleles, the percent recipient cells is equal to R/(R + D), where R is the area under the recipient-specific peak(s) and D is the area under the donor-specific peak(s). Shared alleles, where one allele is the same for donor and recipient (Fig. 11-20) can be dropped from the calculation, and the percent recipient cells is calculated as

$$\frac{R_{(unshared)}}{(R_{(unshared)} + D_{(unshared)})}$$

Chimerism/engraftment results are reported as percent recipient cells and/or percent donor cells in the bone marrow, blood, or cell fraction. These results do not reflect the absolute cell number, which could change independently of the donor/recipient ratio. Inability to detect donor or recipient cells does not mean that that cell population is completely absent, as capillary electrophoresis and fluorescent detection methods offer a sensitivity of 0.1%–1% for autosomal STR markers. Time trends may be more important than single-point results following transplantation.

Because cell lineages engraft with different kinetics, testing of blood and bone marrow may yield different levels of chimerism. Bone marrow will contain more myeloid cells, and blood will contain more lymphoid cells. The first determination to be made from engraftment testing is whether donor engraftment has occurred and secondly whether there is **mixed chimerism**. In mixed chimerism, cell separation techniques may be used to determine which lineages are mixed and which are in fully donor. Nonmyeloablative conditioning of the transplant recipients requires monitoring of both myeloid and lymphoid cell engraftment. This information may be determined by positive or negative lineage separation of whole blood (see the Advanced Concepts box that discusses cell lineage) or by testing blood and bone marrow.

Linkage Analysis

Because the locations of many STRs in the genome are known, these structures can be used to map genes, especially those genes associated with disease. Three basic

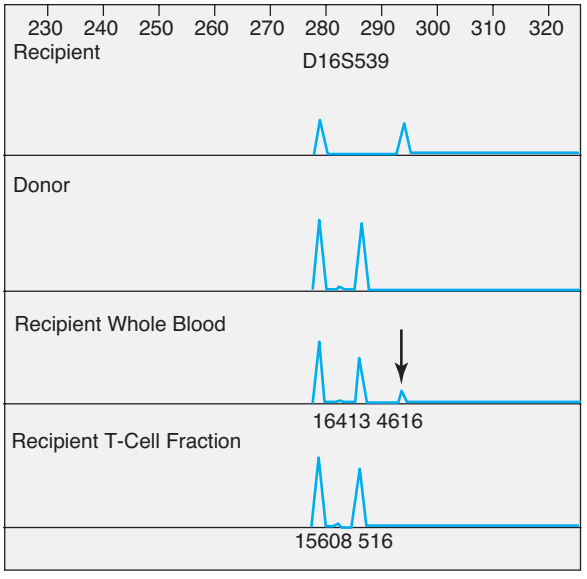


Figure 11-21 Postengraftment analysis of an informative locus D16S539. The area (fluorescence units) under the peaks is calculated automatically. The recipient and donor patterns are shown in the first and second trace, respectively. Results from the whole blood and T-cell fraction are shown in the third and fourth traces. For D16S539 the formula, $R_{(unshared)}/(R_{(unshared)} + D_{(unshared)}$, yields $4616/(4616 + 16413) \times 100 = 22\%$ recipient cells in the unfractionated blood (arrow) and $516/(516 + 15608) \times 100 = 3.3\%$ recipient cells in the T-cell fraction.

approaches are used to map genes, family histories, population studies, and sibling analyses.

Family history and analysis of generations of a single family for the presence of a particular STR allele in affected individuals is one way to show association. Family members are tested for several STRs, and the alleles are compared between affected and unaffected members of the family. Assuming normal mendelian inheritance, if a particular allele of a particular locus is always present in affected family members, that locus must be closely linked to the gene responsible for the phenotype in those individuals (Fig. 11-22). If the linkage is close enough to the gene (no recombination between the STR and the disease gene), the STR may serve as a convenient target for disease testing. Instead of testing for mutations in the disease gene, the marker allele is determined. It is easier, for example, to look for a linked STR allele than to screen a large gene for point mutations. The presence of the "indicator" STR allele serves as a genetic marker for the disease (Fig. 11-23).

Another approach to linkage studies is association analysis in large numbers of unrelated individuals in population studies. Just as with family history studies, close linkage to specific STR alleles supports the genetic proximity of the disease gene with the STR. In this case, however, large numbers of unrelated people are tested for

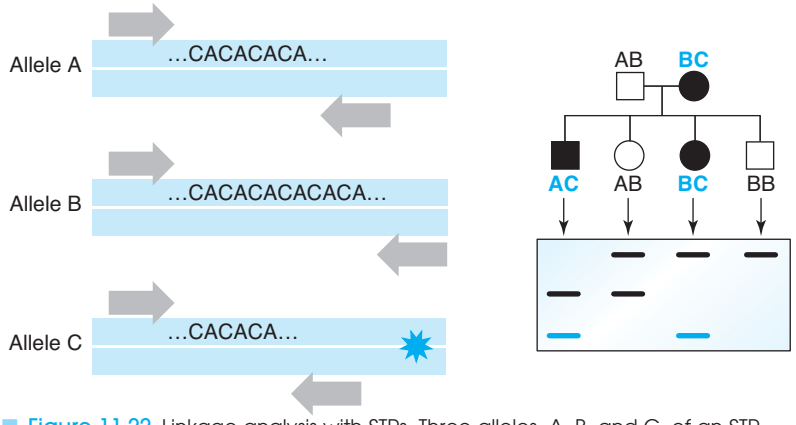
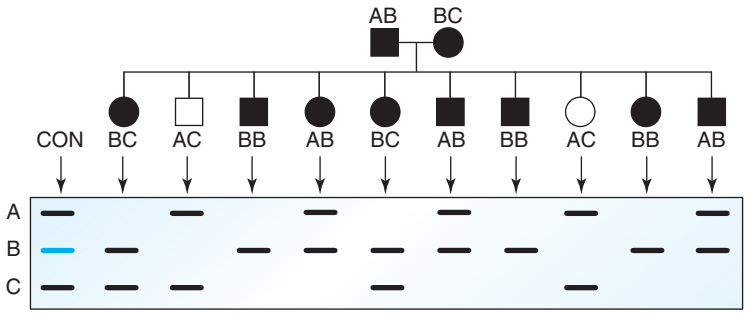


Figure 11-22 Linkage analysis with STRs. Three alleles, A, B, and C, of an STR locus are shown (left). At right is a family pedigree showing assortment of the alleles along with gel analysis of PCR amplification products. Allele C is present in all affected family members. This supports the linkage of this STR with the gene responsible for the disease affecting the family. Analysis for the presence of allele C may also provide a simple indicator to predict inheritance of the affected gene.



■ Figure 11-23 Inheritance of alleles in an affected family. Using the banding pattern shown, the B allele of this STR is always present in affected individuals. This locus must be closely linked to the mutated gene.

linkage rather than a limited number of related individuals in a family. The results are expressed in probability terms that an individual with the linked STR allele is likely to have the disease gene.

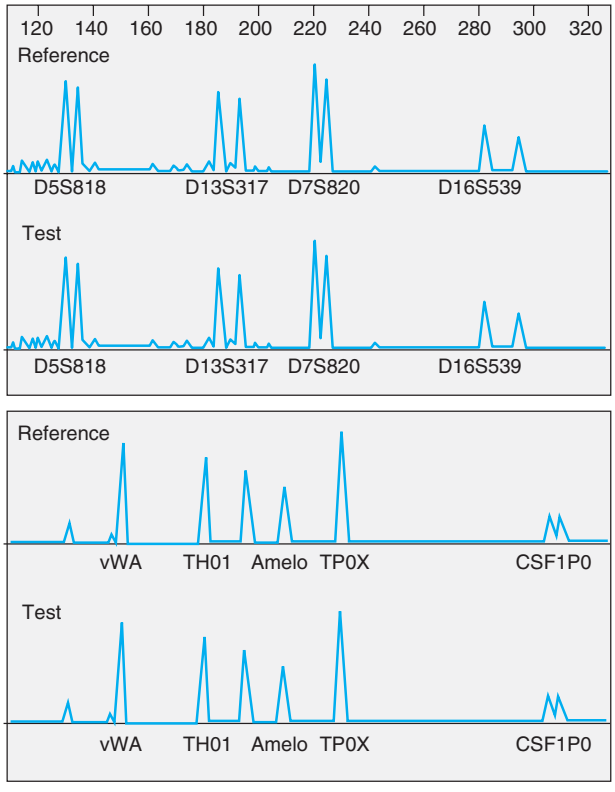
Sibling studies are the third approach to linkage studies. Monozygotic (identical) and dizygotic (fraternal) twins provide convenient genetic controls for genetic and environmental studies. Monozygotic twins will always have the same genetic alleles, including disease genes. There should be 100% recurrence risk (likelihood) that if one twin has a genetic disease, the other twin has it, and both should have the same linked STR alleles. Fraternal twins have the same likelihood of sharing a gene allele as any sibling pair. Investigation of adoptive families may also distinguish genetic from environmental or somatic effects.

Quality Assurance for Surgical Sections Using STR

Personnel in the molecular diagnostics laboratory can assist in assuring that surgical tissue sections are properly identified and uncontaminated. During processing of tissue specimens, microscopic fragments of tissue may persist in paraffin baths (floaters). These fragments can adhere to subsequent slides, resulting in anomalous appearance of the tissue under the microscope. If a tissue sample is questioned, STR identification can be used to confirm the origin of tissue.

For this procedure, reference DNA isolated from the patient and the tissue in question on the slide is subjected to multiplex PCR. The results are compared for matching alleles. If the tissue in question originated from the patient, all alleles should match. Assuming good-quality data, one nonmatching locus excludes the tissue in question as coming from the patient.

An example of such a case is shown in Figure 11-24. A uterine polyp was removed for microscopic examina-



■ Figure 11-24 Quality assurance testing of a tissue fragment.The STR profile of the fragment in question (test) was compared with that of reference DNA from the patient. The alleles matched at all loci, supporting genotypic identity.

tion. An area of malignant tissue was present on the slide. The pathologists were suspicious about the malignancy as there was no other malignant tissue observed in other sections. The tissue fragment was microdissected from the thin section and tested at nine STR loci. The allelic profile was compared to reference DNA from the patient. The profiles were identical, confirming that the tissue fragment was from the patient.

Single Nucleotide Polymorphisms

Data from the Human Genome Project revealed that the human nucleotide sequence differs every 1000–1500 bases from one individual to another.[30] The majority of these sequence differences are variations of single nucleotides or **SNPs**. The traditional definition of polymorphism requires that the genetic variation is present at a frequency of at least 1% of the population. The International SNP Map Working Group observed that two haploid genomes differ at 1 nucleotide per 1331 bp.[31] This rate, along with the theory of neutral changes expected in the human population, predicts 11 million sites in a genome of 3 billion bp that vary in at least 1% of the world's population. In other words, each individual has 11 million SNPs.

Initially, the only way to detect SNPs was by direct sequencing. A number of additional methods have now been designed to detect single nucleotide polymorphisms (see Chapter 9). Computer analysis is also required to confirm that the population frequency of the SNPs meets the requirements of a polymorphism. So far, approximately 5 million SNPs have been identified in the human genome. Almost all (99%) of these have no biological effect. Over 60,000, however, are within genes, and some are associated with disease. A familiar example is the single nucleotide polymorphism responsible for the formation of hemoglobin S in sickle cell anemia. SNPs have been classified according to location with relation to coding sequences and whether they cause a conservative or nonconservative sequence alteration (Table 11.9).

Due to the density of SNPs across the human genome, these polymorphisms were of great interest for genetic mapping, disease prediction, and human identification. The problem was that detection of single base pair changes was not as easy as detection of STRs, VNTRs, or even RFLPs. With improving technology (see methods described in Chapter 9), mapping studies are achieving denser coverage of the genome.[32]

Table 11.9 **Types of SNP**

SNP	Region	Alteration
Type I	Coding	Nonconservative
Type II	Coding	Conservative
Type III	Coding	Silent
Type IV	Noncoding 5′ UTR*	
Type V	Noncoding 3′ UTR	
Type VI	Noncoding, other	

*Untranslated region

In 1999 the SNP Consortium (TSC) was established as a public resource of SNP data. The original goal of TSC was to discover 300,000 SNPs in 2 years, but the final results exceeded 1.4 million SNPs released into the public domain by the end of 2001. Although STRs have had the most practical use in clinical applications, SNPs, with their denser coverage of the genome, are especially attractive markers for future genetic variation and disease association studies.[33,34]

The Human Haplotype Mapping (HapMap) Project

Despite the presence of numerous polymorphisms, any two people are 99.9% identical at the DNA sequence level. Understanding the 0.1% difference is important, in part because these differences may be the basis of differences in disease susceptibility and other variations among "normal" human traits. The key to finding the genetic sources of these variations depends on identification of closely linked markers or landmarks throughout the genome. Genes, RFLPs, VNTRs, STRs, and other genetic structures have been mapped previously; however, long stretches of DNA sequence are yet to be covered with high density. Closely linked markers allow accurate mapping of regions associated with phenotypic characteristics.

Blocks of closely linked SNPs on the same chromosome tend to be inherited together; that is, recombination

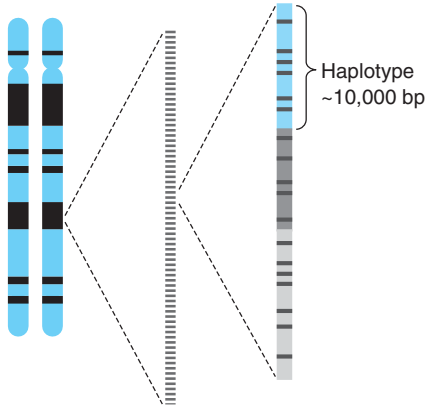


Figure 11-25 Sections of DNA along chromosomes can be inherited as a unit or block of sequence in which no recombination occurs within the block. All the SNPs on that block comprise a haplotype.

rarely takes place within these sequences. This is a phenomenon known as **linkage disequilibrium**. The groups of SNPs comprise **haplotypes**. In the human genome, SNP haplotypes tend to be approximately 20–60,000 bp of DNA sequence containing up to 60 SNPs (Fig. 11-25). Furthermore, as all of the SNPs in the haplotype are inherited together, the entire haplotype can be identified by only a subset of the SNPs in the haplotype. This means that up to 60,000 bp of sequence can be identified through detection of four or five informative SNPs, or **tag SNPs.**[35]

SNP haplotypes offer great potential for mapping of disease genes. A mutation responsible for a genetic disease originally occurs in a particular haplotype, the **ancestral haplotype**. Over several generations the disease allele and the SNPs closest to it (the haplotype) tend to be inherited as a group. This haplotype, therefore, should always be present in patients with the disease. The genetic location and the identification of any disease gene can thus be ascertained, by association with an SNP haplotype. There is, therefore, much interest in developing a **haplotype map** of the entire human genome.

To this end, the Human Haplotype Mapping project was initiated in October, 2002, with a target completion date of September 2005.[36,37] The goal of the project is to map the common patterns of SNPs in the form of a haplotype map, or HapMap. An initial draft of the HapMap was completed before the deadline date, and a second phase was started to generate an even more detailed map. The new phase will increase the density of SNP identification fivefold from 1 SNP per 3000 bases to 1 SNP per 600 bases, or a total of 4.6 million SNPs.

Finding a haplotype frequently in people with a disease, especially genetically complex diseases such as asthma, heart disease, type II diabetes, or cancer, identifies a genomic region that may contain genes contributing to the condition. Because the second phase (phase II HapMap) will be so detailed, the results are expected to advance efforts significantly to locate specific genes involved in these complex genetic disorders.

To create the HapMap, DNA was taken from blood samples from 270 volunteer donors from Chinese, Japanese, African, and European populations. SNPs were detected in DNA from each individual and compared. SNP detection is performed by high throughput detection systems such as Beadarray, Invader, Multiplex Inversion Probe, Fluorescent Polarization-Template Directed Dye Terminator Incorporation, and Homogenous Mass EXTEND (MassArray, Sequenom). See Chapter 9 for descriptions of these assay methods. Ultimately the haplotypes, identified by tag SNPs, will be used for association studies assuming the common disease/common variant hypothesis. That is, diseases will be identified by a pattern of haplotypes in an individual. This information will lead to therapeutic strategies or prediction of treatment response. In addition, genetic determinants of normal traits such as longevity or disease resistance may also be uncovered. Laboratory testing for these haplotypes will be relatively simple to perform and interpret, compared with the more complex methods, such as single-strand conformational polymorphism, that are required to screen for gene mutations. Technologies such as Invader and PyroSequencing are ideally suited to detect known single base changes such as tag SNPs.

Mitochondrial DNA Polymorphisms

Mitochondria contain a circular genome of 16,569 base pairs. The two strands of the circular mitochondrial DNA (mtDNA) chromosome have an asymmetric distribution of Gs and Cs generating a G-rich **heavy** (H)- and a C-rich **light** (L)-strand. Each strand is transcribed from

a control region starting at one predominant promoter, P_L on the L strand and P_H on the H strand, located in sequences of the mitochondrial circle called the displacement (D)-loop (Fig. 11-26). The D-loop forms a triple-stranded region with a short piece of H-strand DNA, the 7S DNA, synthesized from the H strand. P_L starts bidirectional transcription on the L-strand and P_{H1} and P_{H2} on the H-strand. RNA synthesis proceeds around the circle in both directions. A bidirectional attenuator sequence limits L-strand synthesis and, in doing so, maintains a high ratio of rRNA to mRNA transcripts from the H-strand (see Fig. 11-26). The mature RNAs, 1 to 17, are generated by cleavage of the polycistronic (multiple gene) transcript at the tRNAs.

Genes encoded on the mtDNA include 22 tRNA genes, 2 ribosomal RNA genes, and 12 genes coding for components of the oxidation-phosphorylation system. Mutations in these genes are responsible for neuropathies and myopathies (see Chapter 13).

In addition to coding sequences, the mitochondrial genome has two noncoding regions that vary in DNA sequence and are called **hypervariable regions I and II**, **HVI** and **HVII** (see Fig. 11-26). The reference mtDNA hypervariable region is the sequence published initially by Anderson, called the **Cambridge Reference Sequence**, the **Oxford sequence**, or the **Anderson sequence**.[38] Polymorphisms are denoted as variations from the reference sequence. Nucleotide sequencing of the mtDNA control region has been validated for the genetic characterization of forensic specimens[39] and disease states[40,41] and for geneology studies.[42,43]

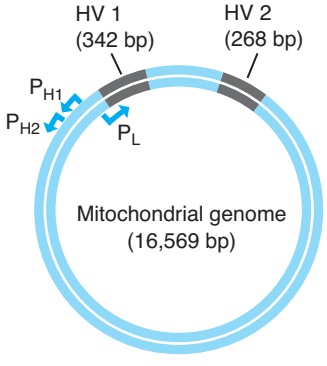


Figure 11-26 The mitochondrial genome is circular. The hypervariable (HV) sites in the control region are shown. Mitochondrial genes are transcribed bidirectionally starting at promoters (P_L and P_H).

In contrast to nuclear DNA, including the Y chromosome, mtDNA follows maternal clonal inheritance patterns. With few exceptions,[44] mtDNA types (sequences) are inherited maternally. These characteristics make possible collection of reference material for forensic analysis, even in cases in which generations are skipped. For forensic purposes, the quality of an mtDNA match between two mtDNA sources is determined by counting the number of times the mtDNA profile occurs in data collections of unrelated individuals. The estimate of uniqueness of a particular mtDNA type depends on the size of the reference database.[39] As more mitochondrial DNA sequences are entered into the database, the more powerful the identification by mitochondrial DNA will become.

Mitochondrial nucleotide sequence data are divided into two components, forensic and public. The forensic component consists of anonymous population profiles and is used to assess the extent of certainty of mtDNA identifications in forensic casework. All forensic profiles include, at a minimum, a sequence region in HVI (nucleotide positions 16024–16383) and a sequence region in HVII (nucleotide positions 53–372). These data are searched through the CODIS program in open case files and missing persons cases. Approximately 610 bp, including the hypervariable regions of mtDNA, are routinely sequenced for forensic analysis. Deviations from the Cambridge reference sequence are recorded as the number of the position and a base designation. For example, a transition from A to G at position 263 would be recorded as 263 G.

The public data consist of mtDNA sequence data from the scientific literature and the GenBank and European Molecular Biology Laboratory databases. The public data have not been subjected to the same quality standards as the forensic data. The public database provides information on worldwide population groups not contained within the forensic data and can be used for investigative purposes.

As all maternal relatives share mitochondrial sequences, the mtDNA of sisters and brothers or mothers and daughters will exactly match in the hypervariable region in the absence of mutations. The use of mtDNA

Advanced Concepts

Mitochondrial profiles in both public and private data sets are identified in a systematic naming scheme. A standard 14-character nucleotide sequence identifier is assigned to each profile. The first three characters indicate the country of origin. The second three characters describe the group or ethnic affiliation to which a particular profile belongs. The final six characters are sequential acquisition numbers. For example, profile USA.ASN.000217 designates the 217th nucleotide sequence from an individual of Asian American ethnicity. The population/ethnicity codes for indigenous peoples are numeric and arbitrarily assigned. For example, USA.008.000217 refers to an individual from the Apache tribe sampled from the United States.

polymorphisms is for exclusion. There is an average of 8.5 nucleotide differences between mtDNA sequences of unrelated individuals in the hypervariable region. The Scientific Working Group for DNA Methods (SWGDAM) has accumulated a database of more than 4100 mtDNA sequences. The size of this database dictates the level of certainty of exclusion using mtDNA.

SWGDAM has recommended guidelines for the use of mtDNA for identification purposes.[45] The process begins with visual inspection of the specimen. Bone or teeth specimens are examined and ascertained to be of human origin. In the case of hair samples, the hairs are examined microscopically and compared with hairs from a known source. Sequencing is performed only if the specimen meets the criteria of origin and visual matching to the reference source. Before DNA isolation, the specimens are cleaned with detergent or, for bone or teeth, by sanding to remove any possible source of extraneous DNA adhering to the specimen. The cleaned specimen is then ground in an extraction solution. Hair shafts yield mtDNA as do the fleshy pulp of teeth or bone. The dentin layer of old tooth samples will also yield mtDNA. DNA is isolated by organic extraction (see Chapter 4) and amplified by PCR (see Chapter 7). The PCR products are then purified and subjected to dideoxy sequencing (see Chapter 10). A positive control of a known mitochondrial sequence is included with every run along with a reagent blank for PCR contamination and a negative control for contamination during the sequencing reaction. If the negative or reagent blank controls yield sequences similar to the specimen sequence, the results are rejected. Both strands of the specimen PCR product must be sequenced.

The mitochondrial sequence traces are imported into a software program for analysis. With the sequence software, the heavy-strand sequences should be reverse-complemented so that the bases are aligned in the light-strand orientation for strand comparison and base designation. Occasionally, more than one mtDNA population is present in the same individual. This is called **heteroplasmy**. In **point heteroplasmy**, two DNA bases are observed at the same nucleotide position. **Length heteroplasmy** is typically a variation in the number of bases in tracts of like bases (homopolymeric tracts, e.g., CCCCC). A length variant alone cannot be used to support an interpretation of exclusion.[46]

Samples cannot be excluded as originating from the same source just on the basis of a sequence matching. The conclusion that an individual can or cannot be eliminated as a possible source of the mtDNA is reached under conditions defined by the individual laboratory. In addition, evaluation of cases in which heteroplasmy may have occurred is laboratory-defined.

In general, if two or more nucleotide differences occur between the reference and test samples, the reference and test samples can be excluded as originating from the same person or a maternally related person. One nucleotide difference between the samples is interpreted as an inconclusive result. If the test and reference samples show sequence concordance, then the test specimen cannot be excluded as coming from the same individual or maternal relative as the source of the reference sequence.

The mtDNA profile of a reference and test sample that cannot be excluded as possibly originating from the same source can be searched in a population database. Population databases such as the mtDNA population database and CODIS are used to assess the weight of forensic evidence, based on the number of different mitochondrial sequences previously identified. The SWGDAM database contains mtDNA sequence information from more than

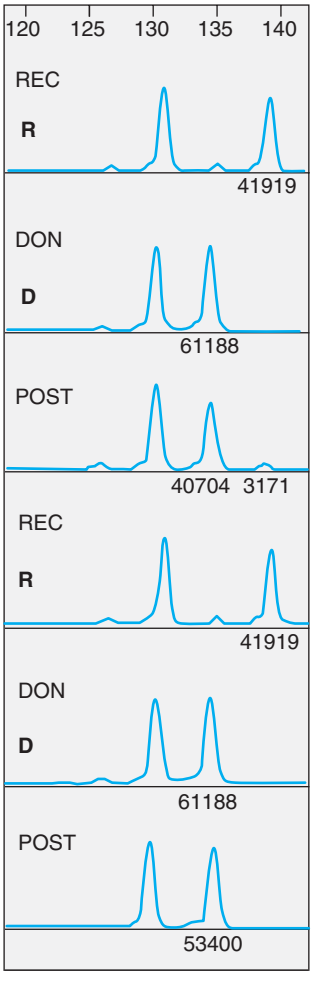
4100 unrelated individuals. The quality of sequence information used and submitted for this purpose is extremely important.[47,48] Based on the number of known mtDNA sequences, the probability of sequence concordance in two unrelated individuals is estimated at 0.003. The probability that two unrelated individuals will differ by a single base is 0.014.

Mitochondrial DNA analysis is also used for lineage studies and to track population migrations. Like the Y chromosome, there is no recombination between mitochondria, and polymorphisms arise mostly through mutation. The location and divergence of specific sequences in the HV regions of mitochondria are an historical record of the relatedness of populations.

Because mitochondria are naturally amplified (hundreds per cell and tens of circular genomes per mitochondria) and because of the nuclease- and damage-resistant circular nature of the mitochondrial DNA, mtDNA typing has been a useful complement to other types of DNA identification. Challenging specimens of insufficient quantity or quality for nuclear DNA analysis may still yield useful information from mtDNA. To this end, mtDNA analysis has been helpful for the identification of missing persons in mass disasters or for typing ancient specimens. MtDNA typing can also be applied to quality assurance issues as described for STR typing of pathology specimens.[49]

Case Study 11 • 1

A 32-year-old woman was treated for mantle cell lymphoma with a nonmyeloablative bone marrow transplant. Before the transplant and after a donor was selected, STR analysis was performed on the donor and the recipient to find informative alleles. One hundred days after the transplant, engraftment was evaluated using the selected STR alleles. The results from one marker, D5S818, are shown in the top panel. One year later, the patient was reevaluated. The results from the same marker are shown in the bottom panel.

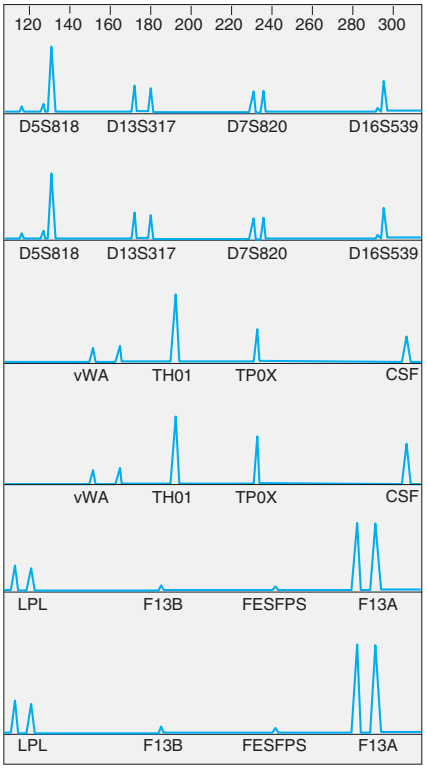


Results from engraftment analysis at 100 days (top) and 1 year (bottom) showing marker D5S818. R. recipient; D, donor.

QUESTION: *Was the woman successfully engrafted with donor cells? Explain your answer.*

Case Study 11 • 2

A young man of 26 years reported to his doctor with joint pain and fatigue. Complete blood count and differential counts were indicative of chronic myelogenous leukemia. The diagnosis was confirmed by karyotyping, showing 9/20 metaphases with the t(9;22) translocation. Quantitative PCR was performed to establish a baseline for monitoring tumor load during and following treatment. Treatment with Gleevec and a bone marrow transplant were recommended. The man had a twin brother, who volunteered to donate bone marrow. The two brothers were not sure if they were fraternal or identical twins. Donor and recipient buccal cells were sent to the molecular pathology laboratory for STR informative analysis. The results are shown below.

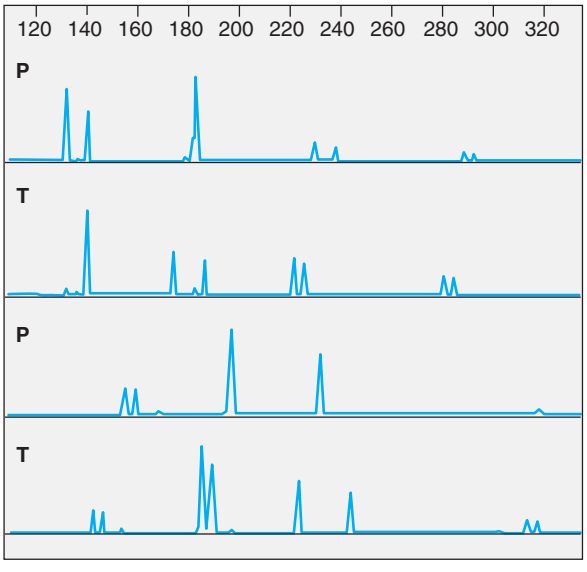


STR analysis of two brothers, one who serves as bone marrow donor (D) to the other (R). Twelve loci are shown.

QUESTION: *Were the brothers fraternal or identical twins? Explain your answer.*

Case Study 11 • 3

A fixed paraffin-embedded tissue section was received in the pathology department with a diagnosis of benign uterine fibroids. Slides were prepared for microscopic study. Only benign fibroid cells were observed on all slides, except one. A small malignant process was observed located between the fibroid and normal areas on one slide. As similar tissue was not observed on any other section, it was possible that the process was a contamination from the embedding process. To determine the origin of the malignant cells, DNA was extracted from the malignant area and compared with DNA extracted from normal tissue from the patient. The results are shown below.



STR analysis of suspicious tissue discovered on a paraffin section. Eight loci were tested. P, patient; T, tissue section.

QUESTION: *Were the malignant cells seen in one section derived from the patient, or were they a contaminant of the embedding process? Explain your answer.*

• STUDY QUESTIONS •

1. Consider the following STR analysis.

Locus	Child	Mother	AF1	AF2
D3S1358	15/15	15	15	15/16
vWA	17/18	17	17/18	18
FGA	23/24	22/23	20	24
TH01	6/10	6/7	6/7	9/10
TPOX	11/11	9/11	9/11	10/11
CSF1PO	12/12	11/12	11/13	11/12
D5S818	10/12	10	11/12	12
D13S317	9/10	10/11	10/11	9/11

   a. Circle the child's alleles that are inherited from the father.
   b. Which alleged father (AF) is the biological parent?

2. The following evidence was collected for a criminal investigation.

Locus	Victim	Evidence	Suspect
TPOX	11/12	12, 11/12	11
CSF1PO	10	10, 9	9/10
D13S317	8/10	10, 8/10	9/12
D5S818	9/11	10/11, 9/11	11
TH01	6/10	6/10, 8/10	5/11
FGA	20	20, 20/22	20
vWA	15/17	18, 15/17	15/18
D3S1358	14	15/17, 14	11/12

The suspect is heterozygous at the amelogenin locus.
   a. Is the suspect male or female?
   b. In the evidence column, circle the alleles belonging to the victim.
   c. Should the suspect be held or released?

3. A child and an alleged father (AF) share alleles with the following paternity index:

Locus	Child	AF	Paternity Index for Shared Allele
D5S818	9,10	9	0.853
D8S1179	11	11,12	2.718
D16S539	13,14	10,14	1.782

   a. What is the combined paternity index from these three loci?
   b. With 50% prior odds, what is the probability of paternity from these three loci?

4. Consider the following theoretical allele frequencies for the loci indicated.

Locus	Alleles	Allele Frequency
CSF1PO	14	0.332
D13S317	9, 10	0.210, 0.595
TPOX	8, 11	0.489, 0.237

   a. What is the overall allele frequency, using the product rule?
   b. What is the probability that this DNA found at the two sources came from the same person?

5. STR at several loci were screened by capillary electrophoresis and fluorescent detection for informative peaks prior to a bone marrow transplant. The following results were observed:

Locus	Donor Alleles	Recipient Alleles
LPL	7, 10	7, 9
F13B	8, 14	8
FESFPS	10	7
F13A01	5, 11	5, 11

   a. Which loci are informative?

6. An engraftment analysis was performed by capillary gel electrophoresis and fluorescence detection. The fluorescence as measured by the instrument under the FESFPS donor peak was 28118 units, and that under

the FESFPS recipient peak was 72691. What is the percent donor in this specimen?

7. The T-cell fraction from the blood sample in Question 6 was separated and measured for donor cells. Analysis of the FESFPS locus in the T-cell fraction yielded 15362 fluorescence units under the donor peak and 97885 under the recipient peak. What does this result predict with regard to T-cell mediated events such as graft-versus-host disease or graft-versus-tumor?

8. If a child had a Y haplotype including DYS393 allele 12, DYS439 allele 11, DYS445 allele 8, and DYS447 allele 22, what are the predicted Y alleles for these loci of the natural father?

9. Which of these would be used for a surname test: Y-STR, mitochondrial typing, or autosomal STR?

10. An ancient bone fragment was found and claimed to belong to an ancestor of a famous family. Living members of the family donated DNA for confirmation of the relationship. What type of analysis would likely be used for this test? Why?

11. What are two biological exceptions to positive identification by autosomal STR?

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Chapter 12

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Detection and Identification of Microorganisms

OUTLINE

OBJECTIVES

- Name the organisms that are common targets for molecular-based laboratory tests.
- Identify advantages and disadvantages of using molecular-based methods as compared with traditional culture-based methods in the detection and identification of microorganisms.
- Differentiate between organisms for which commercially available nucleic acid amplification tests exist and those for which "home-brew" polymerase chain reaction (PCR) is used.
- List the genes involved in the emergence of antimicrobial resistance that can be detected by nucleic acid amplification methods.
- Compare and contrast the molecular methods that are used to type bacterial strains in epidemiological investigations.
- Explain the value of controls, in particular amplification controls, in ensuring the reliability of PCR results.
- Interpret pulse field gel electrophoresis patterns to determine whether two isolates are related to or different from each other.

The use of molecular-based tests in the clinical microbiology laboratory has exploded over the last 10–15 years. A brief review of a recent table of contents of the *Journal of Clinical Microbiology* shows that the majority of research papers that are published in that journal are based on the molecular characterization of microorganisms and the development and evaluation of molecular-based laboratory tests that are used to detect and identify microorganisms in clinical specimens and isolated in cultures. Another important application of molecular technology in the clinical microbiology laboratory is in the comparison of biochemically similar organisms in outbreak situations, known as molecular epidemiology, to ascertain whether the isolates have a common or independent source.

When the potential of molecular-based methods was first realized and the successful amplification of microorganism nucleic acid was first demonstrated, a common fear (or hope, depending on the perspective) of microbiologists was that the detection and identification of microorganisms by traditional culture, stains, and biochemical testing would be relegated to the history books and that molecular-based testing would be the sole methodology in the clinical microbiology laboratory. Although molecular-based methods have definitely found a niche in clinical microbiology, traditional culture and biochemical testing are still the major methods used for the detection and identification of most microorganisms and will continue to be the major method for a long time.

Clinically important microorganisms include a range of life forms from arthropods to prions. In contrast to classical testing that analyzes phenotypic traits of microorganisms (microscopic and colonial morphologies, enzyme or pigment production, as well as carbohydrate fermentation patterns), the analyte for molecular testing is the genome of the microorganism. Bacteria, fungi, and parasites have DNA genomes, whereas viruses can have DNA or RNA genomes. Prions, which cause transmissable encephalopathies such as Creutzfeldt-Jakob disease, consist only of protein.

Microorganisms targeted for molecular-based laboratory tests have been those that are difficult and/or time-consuming to isolate, such as *Mycobacterium tuberculosis* as well as other species of *Mycobacterium*[1-3]; those that are hazardous with which to work in the clinical laboratory, such as *Histoplasma* [4,5] and *Coccidioides*[6,7]; and those for which reliable laboratory tests are lacking, such as Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV).[8]

Additionally, molecular-based tests have been developed for organisms that are received in clinical laboratories in high volumes, such as *Streptococcus pyogenes* in throat swabs and *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in genital specimens.[9] Furthermore, genes that confer resistance to antimicrobial agents are the targets of molecular-based methodologies, such as *mecA*, that contributes to the resistance of *Staphylococcus aureus* to oxacillin[10]; *vanA*, *vanB*, and *vanC,* which give *Enterococcus* resistance to vancomycin[11]; and *katG* and *inhA,* which mediate *M. tuberculosis* resistance to isoniazid.[12,13]

Finally, characterization (sequencing) of DNA and RNA is being used to find and identify new organisms, such as *Tropheryma whipplei*[14] and also to further characterize or genotype known organisms, such as species of *Mycobacterium*, HCV, and HIV. Nucleic acid sequence information is also used to reclassify bacterial organisms based on 16S rRNA sequence homology, for epidemiological purposes, and to predict therapeutic efficacy.

The molecular methods that are used in the clinical microbiology laboratory are the same as those that were described previously for the identification of human polymorphisms and those that will be discussed in subsequent chapters for the identification of genes involved in cancer and in inherited diseases. The primary molecular methods used in clinical microbiology laboratories are polymerase chain reaction (PCR): traditional, real-time, and reverse transcriptase PCR (see Chapter 7), as well as sequencing (see Chapter 10). An additional method that is used in molecular epidemiology is pulsed-field gel electrophoresis (PFGE) (see Chapter 5) as well as other methods that will be discussed in this chapter. All types of microorganisms serve as targets for molecular-based laboratory tests from bacteria to viruses, fungi, and parasites. But the development of molecular-based methods has been more successful for only some organisms and not yet for all organisms, as will be discussed in this chapter.

Specimen Collection

As with any clinical test, collection and transport of specimens for infectious disease testing can affect analytical results negatively, unless proper procedure is followed.

Microbiological specimens may require special handling to preserve the viability of the target organism. Special collection systems have been designed for collection of strict anaerobes, viruses, and other fastidious organisms. Although viability is not as critical for molecular testing, the quality of nucleic acids may be compromised if the specimen is improperly handled. DNA and especially RNA can be damaged in lysed or nonviable cells. Due to the sensitivity of molecular testing, it is also important to avoid contamination that could yield false-positive results.

Collection techniques designed to avoid contamination from the surrounding environment of adjacent tissues apply to molecular testing, especially by amplification methods. Sampling must include material from the original infection. The time and site of collection must be optimal for the likely presence of the infectious agent. For example, *Salmonella typhi* is initially present in peripheral blood but not in urine or stool until at least 2 weeks after infection. For classical methods that include culturing of the agent, a sufficient number of microorganisms ($>10^3$/mL specimen) must be obtained for agar or liquid culture growth. For molecular testing, however, minimum numbers (as few as 50 organisms) can be detected successfully. The quantity of target organisms as well as the clinical implications should be taken into account when interpreting the significance of positive results, as molecular detection can reveal infective agents at levels below clinical significance.

Equipment and reagents used for specimen collection are also important for molecular testing (Table 12.1). Blood draws should go into the proper anticoagulant, if one is to be used. (See Chapter 16 for a list of anticoagulants and their effect on molecular testing.) Although wooden-shafted swabs may be used for throat cultures, dacron or calcium alginate swabs with plastic shafts are recommended for collection of bacteria, viruses, and mycoplasma from mucosal surfaces.[15] The plastics are less adherent to the microorganisms and will not interfere with PCR reagents as do emanations from wooden-shafted swabs. The swab extraction tube system (SETS, Roche Diagnostics) is designed for maximum recovery of microorganisms from swabs by centrifugation.

Commercial testing kits supply an optimized collection system for a particular test organism. The Clinical and Laboratory Standards Institute has published documents addressing the requirements for transport devices and quality control guidelines. The College of American Pathologists requires documented procedures describing specimen handling, collection, and transport in each laboratory.

Table 12.1 **Specimen Transport Systems**

Type	Examples
Sterile containers	Sterile cups, screw-capped tubes, stoppered tubes, Petri dishes
Swabs	Calcium alginate swabs, Dacron swabs, cotton swabs, nasopharyngeal-urogenital swabs, Swab Transport System
Specialty systems	*N. gonorrhoeae* transport systems, SETS.
Proprietary systems	Molecular testing, *Neisseria gonorrhoeae* transport systems, STAR buffer[42]
Anaerobic transport systems	Starplex Anaerobic Transport system (Fisher), BBL Vacutainer Anaerobic Specimen Collector
Viral transport systems	BD Cellmatics Viral Transport Pack, BBL Viral Culturette

Advanced Concepts

Biological safety is an important concern for clinical microbiology. Because various collection, transport, and extraction systems inactivate organisms at different times, the technologist should follow recommendations of the Centers for Disease Control and Prevention (CDC) that call for universal precautions, treating all specimens as if they were infectious throughout the extraction process. Updated guidelines are available from the CDC for the handling of suspected bioterrorism material; for example, the anthrax spores discovered in the United States Postal System in 2001. Organisms such as smallpox must be handled only in approved (level 4 containment) laboratories. Molecular testing has eased the requirements for preserving the viability of organisms for laboratory culture. This should improve safety levels as methods are devised that replace growing cultures.

Sample Preparation

The isolation of nucleic acids was described in Chapter 4. Isolating nucleic acids from microorganisms is similar to isolating nucleic acids from human cells with only a few additional considerations. First, depending on the microorganism, more rigorous lysis procedures may be required. Mycobacteria and fungi in particular have thick cell walls that are more difficult to lyse than those of other bacteria and parasites. Gram-positive bacteria having a thicker cell wall than gram-negative bacteria may require more rigorous cell lysis conditions. *Mycoplasma*, on the other hand, lacks a cell wall, and so care must be taken with the sample to avoid spontaneous lysis of the cells and loss of nucleic acids. Chapter 4 has a complete description of cell lysis methods.

Second, the concentration of organisms within the clinical sample must be considered. Samples should be centrifuged to concentrate the fluid and the organisms within the fluid from the milliliters of sample that are often received, down to microliter volumes that are used in nucleic acid amplification procedures. Third, inhibitors of enzymes used in molecular analysis may be present in clinical specimens; removal or inactivation of inhibitors must be a part of specimen preparation. Finally, if RNA is to be analyzed, inactivation or removal of RNases in the sample and in all reagents and materials that come into contact with the sample must occur.

Any clinical specimen can be used as a source of microorganism nucleic acid for analysis. Depending on the specimen, however, special preparation procedures may be necessary to allow for optimal nucleic acid isolation, amplification, and analysis. In cerebrospinal fluid, inhibitors of DNA polymerase have been demonstrated; therefore, careful isolation of nucleic acid from other molecules present in the sample will more likely result in an amplifiable sample. Isolation of nucleic acids from blood was discussed in Chapter 4. When processing a whole blood specimen, it is critical to remove all of the hemoglobin and other products of metabolized hemoglobin because they have been shown to be inhibitors of DNA polymerase and thus may prevent the amplification of nucleic acid in the sample, resulting in a false negative. White blood cells, such as lymphocytes, can be used as a source of nucleic acid for organisms, primarily viruses that infect these cells. In this case, the cells are isolated from the red blood cells using Ficoll-Hypaque and then lysed. Serum or plasma can also be used as a source of microorganism nucleic acid.

Sputum is used as a source of nucleic acid from organisms that cause respiratory tract infections. Acidic polysaccharides present in sputum may inhibit DNA polymerase and thus must be removed. Using a method that reliably separates DNA from other cellular molecules is sufficient in removing the inhibitors. Urine, when sent for nucleic acid isolation and amplification, is treated similarly to cerebrospinal fluid; i.e., the specimen is centrifuged to concentrate the organisms and then subjected to nucleic acid isolation procedures. Inhibitors of DNA polymerase, namely nitrate, crystals, hemoglobin, and beta-human chorionic gonadotropin, have been demonstrated in urine as well.[16]

The type of specimen sent for molecular testing may also affect extraction and yield of nucleic acid. For example, viral nucleic acid from plasma is easier to isolate than nucleic acid from pathogenic *Enterococcus* in stool specimens. Reagents and devices have been developed to combine collection and extraction of nucleic acid from difficult specimens; for example, stool transport and recovery (STAR, Roche Diagnostics) buffer or the FTA paper systems that inactivate infectious agents and adhere nucleic acids to magnetic beads or paper, respectively.

Quality Control

Quality control for any clinical laboratory procedure is critical for ensuring the accuracy of patient results, and ensuring the quality of molecular methods in the clinical microbiology laboratory is equally important. The sensitivity of molecular methods is so high that even one molecule of target can be used as a template. Thus, ensuring that the integrity of specimens is maintained, i.e., that specimens are not contaminated by other specimens or with the products of previous amplification procedures, is critical to avoid false positives. On the other hand, it is equally important to ensure that the lack of a product in an amplification procedure is due to the absence of the target organism and not the presence of inhibitors preventing the amplification of target sequences (false negative).

The incorporation of **positive controls** in a nucleic acid amplification assay shows that the assay system is functioning properly. A **sensitivity control** that is positive at the lower limit of detection demonstrates sensitivity of

qualitative assays. Two positive controls, one at the lower limit and the other at the upper limit of detection, should be run in quantitative assays to test the dynamic range of the assay. **Reagent blank** or **contamination controls** are critical for monitoring reagents for carry-over contamination. These controls contain all of the reagents except target sequences and should always be negative. For typing and other studies that might include nontarget organisms, a **negative template control** containing nontarget organism(s) should also be included. With regard to amplification controls (see below), the negative template control should have a positive amplification control signal, whereas the reagent blank should be negative for target and amplification. The presence of an amplicon in the negative control negates the assay, and the source of the contamination must be found.

In order to rule out false negatives due to amplification failure, an **amplification control** aimed at a target that is always present can be incorporated into an amplification assay. If the amplification control is amplified, then the fact that the target did not amplify can be more confidently interpreted as a true negative result. Amplification controls are usually housekeeping genes or those that are always present in a human sample. Housekeeping genes that are used as internal controls include prokaryotic genes such as *groEL*, *rpoB*, *recA*, and *gyrB*[19] and eukaryotic genes such as β-actin, glyceraldehyde-3-phosphate, interferon-γ, extrinsic homologous control, human mitochondrial DNA and peptidylprolyl isomerase A.[15,20]

Internal controls are amplification controls that monitor particular steps of an amplification method. Internal controls can be either **homologous extrinsic**, **heterologous extrinsic,** or **heterologous intrinsic**. A homologous extrinsic control is a wild-type–derived control with a nontarget-derived sequence insert. This control is added to every sample after nucleic acid extraction and before amplification. The amplification of this control occurs using the same primers as for the target. It is good for ensuring that amplification occurs in the sample, but it does not control for target nucleic acid degradation during extraction. Heterologous extrinsic controls are nontarget-derived controls that are added to every sample before nucleic acid extraction. This control will ensure that extraction and amplification procedures were acceptable, but a second set of primers must also be added to the reaction for this control to be amplified. Use of this control requires that the procedure be optimized such that the amplification of the control does not interfere with the amplification of the target.

Heterologous intrinsic controls are eukaryotic genes. Human gene controls serve to ensure that human nucleic acid is present in the sample in addition to controlling for extraction and amplification. The use of this control requires that either two amplification reactions are performed on the sample, one for the control and the other for the target gene, or that the amplification procedure be multiplexed, which may result in interference of the amplification of the target.

In a procedure that detects a microorganism, a positive result states that the organism is present in that sample, whereas a negative result indicates that the organism is not present (at least not at amounts up to the detection limits of the assay). Although most false positives can be eliminated by preventing carryover contamination, another source of false positives that cannot be controlled in the laboratory is the presence of dead or dying microorganisms in the sample of a patient taking antimicrobial agents. In this situation, the nucleic acid–based tests will remain positive longer than culture assays and thus may appear as a false positive. Repeating the nucleic acid–based assay 3–6 weeks after antimicrobial therapy is more likely to yield a true negative result.[15]

False-negative results may be more problematic and arise when the organism is present, but the test result is negative. There are a few reasons for obtaining false-negative results on a sample. First, the organism may be present, but the nucleic acid was degraded during collection, transport, and/or extraction. This can be prevented by proper specimen handling, effective transport media, and inhibiting the activity of DNases and RNases that may be present in the sample and in the laboratory. Second, amplification procedures can be inhibited by substances present in the specimen. Hemoglobin, lactoferrin, heparin and other anticoagulants, sodium polyanethol sulfonate (anticoagulant used in blood culture media), and polyamines have been shown to inhibit nucleic acid amplification procedures.[15] Attention to nucleic acid isolation procedures and ensuring optimal purification of nucleic acid from other components of the specimen and extraction reagents will help minimize the presence and influence of inhibitors on the amplification reaction. Experimenting with different commercial nucleic acid extraction systems may result in discovering a system that is optimal for a particular purpose.[15]

Extensive validation must be performed on new molecular-based tests that are brought into the laboratory (see Chapter 16). Controls must be tested, and the sensitivity, specificity, and reproducibility of the assay must be determined. Proficiency testing of personnel should be performed regularly to ensure that the people performing the tests are doing so correctly. The Clinical and Laboratory Standards Institute,[19] Association for Molecular Pathology,[20] and the Food and Drug Administration (FDA) have guidelines for molecular methods in the laboratory.

Bacterial Targets of Molecular-Based Tests

Selection of Sequence Targets for Detection of Microorganisms

Molecular methods are extremely sensitive and specific, but these qualities are limited by the choice of target sequences for primer or probe hybridization. The primary nucleotide sequence of many clinically important microorganisms is now known. Sequences are available from GenBank or from published literature. The specificity of molecular methods targeting these sequences depends on the primers or probes that must hybridize to the chosen point in the genome of the microorganism.

Choosing a sequence target is critical for the specificity of a molecular test (Fig. 12-1). Many microorganisms share the same sequences in evolutionarily conserved genes. These sequences would not be used for detection of specific strains as they are likely to cross-react over a range of organisms. Sequences unique to the target organism are therefore selected. Some organisms, such as HIV, have variable sequences within the same species. Such variations may be informative, for instance, in determining drug resistance or for epidemiological information; however, not all types would be detected by a single sequence. The variable sequences may be included in the probe or primer areas to differentiate between types. These type-specific probes/primers can be used in a confirmatory test after an initial test using probes or primers directed to a sequence shared by all types.

In addition to their strain- or species-specificity, the target sequences must meet technical requirements for hybridization conditions. Primers should have similar annealing temperatures and yield amplicons of appropriate size. Probes must hybridize specifically under the conditions of the procedure. Sequence differences can be distinguished using sequence-specific probes or primers (see Chapters 6 and 7).

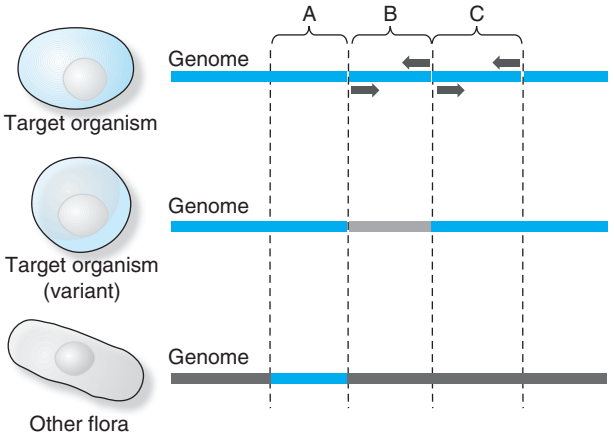


Figure 12-1 Selection of target sequences for a nucleic acid test. The genomes of three organisms, the test target, a variant or different type of the test target, and another nontarget organism, are depicted. Sequence region A is not specific to the target organism and is, therefore, not an acceptable area for probe or primer binding to detect the target. Sequences B and C are specific to the target. Sequence B is variable and can be used to detect and type the target, although some variants may escape detection. Sequence C will detect all types of the target organism, but cannot be used for determining the type.

Probe design includes decisions as to the length of the probe, whether the probe is DNA, RNA, or protein; how the probe is labeled; and, for nucleic acid probes, the length of sequences included in the probe. The source of the probe is also important, as probes must be replenished and perform consistently over long-term use. Probes are manufactured synthetically or biologically by cloning (see Chapter 6). Synthetic oligonucleotides may be preferred for known sequences where high specificity is required. Primer design includes the length and any modifications of the primers and type of signal generation for quantitative PCR. Refer to Chapters 6 and 7 for further discussion of hybridization and amplification methods.

Many tests currently used in molecular microbiology are supplied as commercially designed systems, including prevalidated probes and/or primers. Several of these methods are FDA-approved or FDA-cleared (Table 12.2). An updated list of the currently available FDA-approved tests is available at ampweb.org

Table 12.2 **FDA-Approved/Cleared Test Methods**

Target Organism	Methods*
Cytomegalovirus	NASBA, Hybrid capture
Hepatitis C virus (HCV), qualitative	TMA, PCR
HCV, quantitative	bDNA
Human immunodeficiency virus (HIV), quantitative	bDNA, NASBA, RTPCR
HIV resistance testing	Sequencing
Hepatitis B virus/HCV/HIV screening for blood donations	TMA, PCR, RTPCR
Human papillomavirus	Hybrid capture
Chlamydia trachomatis (CT)	Hybrid capture, TMA, hybridization protection assay, PCR
Neisseria gonorrhoeae (NG)	Hybrid capture, TMA, hybridization protection assay, PCR
CT/NG	Hybrid capture, TMA, hybridization protection assay, PCR, SDA
Gardnerella, Trichomonas vaginalis, Candida	Hybridization
Group A Streptococci	Hybridization protection assay
Group B Streptococci	Hybridization protection assay, real-time PCR
Legionella pneumophila	SDA
Methicillin Resistant *Staphylococcus aureus*	Real-time PCR
Mycobacterium tuberculosis	TMA, PCR

*See Chapters 7 and 9 for detailed description of these methods.

Manufacturers of these commercial reagents provide quality assurance requirements including controls and assay limitations. Each system must be validated on the type of specimen used for clinical testing, including serum, plasma, cerebrospinal and other body fluids, tissue, cultured cells, and organisms. In addition to the commercial reagent sets, many professionals working in clinical laboratories have been developing laboratory protocols (home-brew PCR) for most of the testing that they perform. Primers are designed based on sequence information that has been published; the reagents are bought separately, and the procedures are developed and optimized within the individual laboratory.

Molecular Detection of Bacteria

Molecular-based methods that have been used to detect and identify bacteria include nucleic acid sequence–based amplification (NASBA), Qβ-replicase, and PCR, including the following modifications: real-time or quantitative, reverse transcriptase, nested, and multiplex (see Chapter 7 for explanations of these methods). Product detection is accomplished by a variety of methods including Southern blot hybridization (see Chapter 6), agarose gel electrophoresis (see Chapter 5), PCR (see Chapter 7), sequencing (see Chapter 10), enzyme immunoassay, dot blot hybridization (see Chapter 6), and restriction enzyme analysis (see Chapter 6).

Real-time PCR, or quantitative PCR (qPCR), is used increasingly for detection of infectious agents as it provides the sensitivity of PCR with more information than is available from conventional PCR. The quantitative capability of qPCR allows distinction of subclinical levels of infection (qualitatively positive by conventional PCR) from higher levels with pathological consequences. Furthermore, qPCR programs can be designed to provide closed-tube sequence or typing analysis by adding a melt curve temperature program following the amplification of the target (Fig. 12-2). Like conventional PCR, qPCR can be performed on DNA extracted directly from clinical specimens, including viral, bacterial, and fungal pathogens.

Design of a qPCR method requires selection of a target gene unique to the specimen or specimen type for which primers and probes can be designed. The DNA-specific dye, SYBR Green, can be used in place of probes if the amplicon is free of artifacts such as misprimes or primer dimers (see Chapter 7). Probe types used most often include fluorescent energy transfer hybridization probes and hydrolysis (TaqMan) probes. The require-

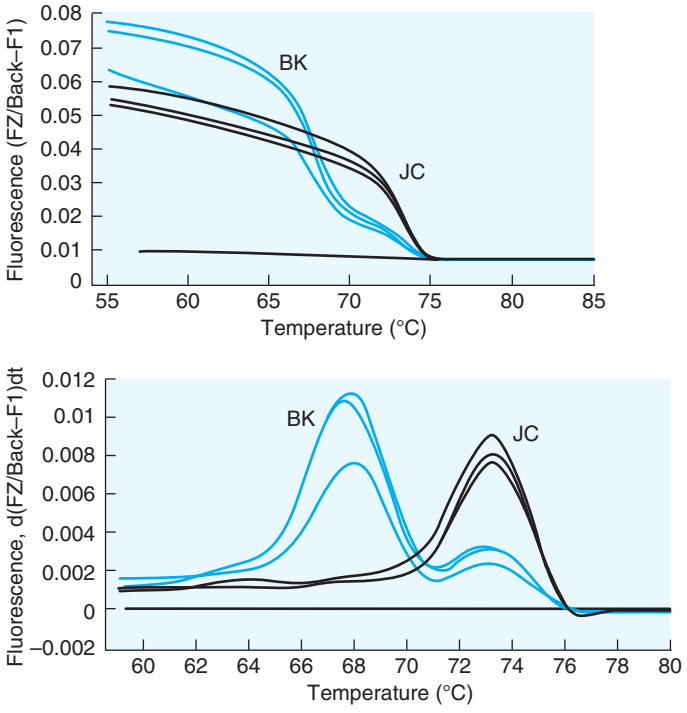


Figure 12-2 Melt curve analysis of BK and JC viruses. BK and JC are differentiated from one another by differences in the T_m^* of mthe probe specific for each viral sequence. Fluorescence from double-stranded DNA decreases with increasing temperature and DNA denaturation to single strands (top panel). Instrument software will present a derivative of the fluorescence (bottom panel) where the T_ms (67°–68°C for BK and 73°–74°C for JC) are observed as peaks.

ment for probes in addition to primers increases the complexity of the design process. Instrument software and several Web sites offer computer programs that automatically design primers and probes on submitted sequences. Commercial primer and probe sets are also available for purchase in kit form. A variety of gene targets have been used for qPCR detection of a number of organisms. A list of examples of targets and probes is available in a comprehensive review by Espy et al.[21]

The genes that have been the targets for the design of primers include ribosomal RNA (rRNA), both 16S and 23S, and housekeeping genes such as *groEL*, *rpoB*, *recA*, and *gyrB*.[21] 16S rRNA is a component of the small subunit of the prokaryotic ribosome, and the 23S rRNA is a component of the large subunit of the prokaryotic ribosome. Analysis of 16S rRNA is performed to determine the evolutionary and genetic relatedness of microorganisms and is driving changes in microorganism nomenclature.[22] The rDNA that encodes the rRNA consists of alternating regions of conserved sequences and sequences that vary greatly from organism to organism. The conserved sequences encode the loops of the rRNA and can be used as a target to detect all or most bacteria. The sequences that have a great amount of heterogeneity encode the stems of the rRNA and can be used to detect a specific genus or species of bacteria.[19] rRNA was the original target of many bacterial molecular-based assays, but because of the instability and difficulty in analyzing RNA, current assays amplify and detect rDNA sequences.

Respiratory Tract Pathogens

Bacteria that cause respiratory tract disease account for significant morbidity and mortality levels around the world. Many of these organisms are endemic even in higher socioeconomic countries and are ubiquitous in the environment. Bacteria in the respiratory tract are easily transmitted by contact with infected respiratory secretions, and laboratory detection and identification by nonmolecular methods often lack sensitivity and/or are time consuming. Because of these organisms' importance in causing human disease and the lack of sensitive, rapid traditional laboratory testing, the development of molecular-based assays that can detect and identify bacterial pathogens directly in respiratory specimens (Table 12.3) has been a priority.

Table 12.3 **Typical Respiratory Tract Organisms Targeted by Molecular-Based Detection Methods**[23, 24]

Organism	Specimen Source	Gene target	Traditional Diagnostic Methods
Mycoplasma pneumoniae	Bronchoalveolar lavage	16S rRNA	Culture
		16S rDNA	Serology
		Species-specific protein gene	
		P1 adhesion gene	
Chlamydophila pneumoniae	Respiratory	Cloned Pst I fragment	Culture
	Throat	16S rRNA	
	Artherosclerotic lesions	MOMP	
Legionella	Deep respiratory secretions	5S rRNA *mip* gene	Culture
	Serum	16S rRNA	Antigen detection
	Buffy coat		
	Urine		
Bordetella pertussis	Nasopharyngeal	IS 481	Culture
		Adenylate cyclase gene	DFA
		Porin gene	
		Pertussis toxin promoter region	
Streptococcus pneumoniae	Blood	DNA polymerase gene	Culture
	CSF	*plyA* (pneumolysin)	
	Serum	*lytA* (autolysin)	
	Sputum	*pbp2a* (penicillin-binding protein)	
		pbp2b	
		pspA (pneumococcal surface protein)	
Mycobacterium tuberculosis	Sputum	16S rRNA	Culture
	Bronchoalveolar lavage		
	Bronchial washings		
	Gastric aspirates		

Mycobacterium tuberculosis

M. tuberculosis is an important cause of respiratory tract infections, is a worldwide problem, and results in infections that have significant morbidity and mortality levels. The diagnosis of tuberculosis can be difficult and can take prolonged periods during which the patient is not adequately treated and may spread the organism to other people. The genome of *M. tuberculosis* has been sequenced and has 4,411,529 bp with about 4000 genes that encode proteins and 50 RNA-encoding genes.[25] More than 250 genes encode enzymes involved in the metabolism of fatty acids, compared with just 50 genes of the same function that are found in *Escherichia coli*. The genome of different isolates of *M. tuberculosis* does not vary to any great extent, and most variation is due to the movement of insertion elements rather than to point mutations.[26]

For many years, the diagnosis of tuberculosis consisted of the performance of mycobacterial smears and culture. Whereas a fluorochrome stain has increased sensitivity compared with the Kinyoun and Ziehl-Neelsen stains for detecting mycobacteria directly in clinical specimens, the sensitivity of smears in general for mycobacteria is 22%–80%.[27] At least 10^4 organisms/mL are required in order to see mycobacteria in a smear, and then only 60% of those smears were read as positive.[28] Cultures for *M. tuberculosis* are more sensitive than smears, able to detect 10^1–10^2 organisms/mL of specimen, but they are still problematic because the organism grows very slowly in vitro and may not be isolated for 2–3 weeks after specimen collection.[29] Liquid-based culture systems have improved the detection rate of mycobacteria to a few days, depending on the organism load.

Once mycobacteria are detected growing in a culture, they must be speciated. The traditional method (and still the only method for most mycobacterial species) for speciation is biochemical testing, which can take a few weeks to perform. Mycolic acid analysis by high performance liquid chromatography has been used by some laboratory professionals to identify mycobacterial species either in a smear-positive specimen or from the growth in liquid or on solid media,[30] but it is not performed in most laboratories.

Mycobacterial identification was revolutionized with the development of DNA probe assays by GenProbe, Inc. and their implementation in clinical mycobacteriology laboratories in 1990. The AccuProbe family of tests is available for the identification of the following species of *Mycobacterium: tuberculosis* complex (*tuberculosis*, *bovis*, and *africanum*), *avium-intracellulare*, *kansasii*, and *gordonae*. The AccuProbe tests detect mycobacterial-specific sequences of 16S rRNA when the rRNA forms a hybrid complex with reagent probe DNA. The hybrid rRNA-DNA complexes are detected in a luminometer that measures chemiluminescence given off by the acridinium ester attached to the DNA probe. The AccuProbe assay can be performed on colonies growing on solid media or from the growth in liquid media. Combining isolation of mycobacteria in a liquid-based medium with identification of species using AccuProbe has decreased the detection and identification of *M. tuberculosis* in particular by at least 3 weeks.[31]

Although DNA probes greatly simplified and reduced the time involved in identifying mycobacterial species as compared with traditional biochemical testing, their sensitivity was not great enough so that they could be used to detect mycobacteria directly in a clinical specimen. The advent of nucleic acid amplification methodologies led to the development of laboratory tests in which *M. tuberculosis* could be detected directly in a clinical specimen with reliable sensitivity and specificity. Two such tests are available: the Amplified *M. tuberculosis* Direct Test (MTD; GenProbe, Inc., San Diego, CA) and the AMPLICOR *M. tuberculosis* PCR test (AMPLICOR MTB; Roche Diagnostics Systems, Branchburg, NJ)

GenProbe's MTD test is FDA-approved for the direct detection of *M. tuberculosis* in smear-positive and -negative respiratory tract samples. The assay can be performed on nonrespiratory samples with slight modification, although it is not yet FDA-approved for this use. The MTD test uses transcription-mediated amplification (see Chapter 7) to amplify 16S rRNA present in a concentrated clinical sample. The amplified rRNA is detected using the same DNA probe as that used in the DNA probe assays described above and measuring chemiluminescence of rRNA-DNA hybrids. The sensitivity of the MTD when compared with smear and culture of smear-positive respiratory samples was 100% and 83% for specimens that had a negative smear.[32] Amplification and detection can be performed in 3.5 hours in a single tube and have 100% specificity for *M. tuberculosis* complex. The MTD test is subject to false negatives when inhibitors are present in the clinical sample as well as when only a few organisms are present in the sample. The incorporation of internal controls and ensuring the amplification of the control in a valid test help to decrease the likelihood of false-negative samples.[32]

The AMPLICOR MTB test is a kit-based PCR assay for the detection of *M. tuberculosis* complex directly in a clinical specimen.[33] Cells are lysed in the sample, releasing mycobacterial DNA, DNA is denatured, primers complementary to a 584-bp region of 16S rRNA that is common to all mycobacteria hybridize to target sequences, and DNA polymerase makes a copy of the target DNA. dUTP and uracil-N-glycosylase are added to prevent carryover contamination. Product detection is accomplished using a DNA probe specific for *M. tuberculosis* complex and avidin–horseradish peroxidase conjugate–tetramethyl benzidine substrate system. The AMPLICOR MTB test takes 6.5–8 hours to complete and has a sensitivity of 55.3% in smear-negative samples and 94.7% sensitivity in smear-positive samples.[33] The difference in sensitivities reflects the importance of organism burden in the sample in yielding a positive, even in a highly sensitive assay such as PCR.

In a direct comparison of MTD and AMPLICOR MTB assays, both assays agreed with culture and clinical diagnosis in 96.8% of the samples.[34] The MTD test had a better sensitivity of 95.9 when compared with the AMPLICOR MTB assay (85.4), which was statistically significant ($p = 0.045$). The specificity of both assays was comparable with a specificity of the MTD assay of 98.9 and 99.6 for the AMPLICOR MTB.

Bordetella pertussis

B. pertussis is an upper-respiratory tract pathogen that is the causative agent of whooping cough. The organism is

endemic worldwide and is transmitted via direct contact with infected respiratory secretions. Children 1–5 years of age are the primary targets of *B. pertussis*. In 1947 vaccination against *B. pertussis* was implemented in the United States, and cases of whooping cough in the target age group decreased significantly. Infections in infants younger than 1 year of age increased, however, after the vaccination program was started because the infants had not received the full series of three shots.[25] Recently, outbreaks of pertussis have been identified in adolescents and adults whose immunity owing to the vaccine waned. Infections in these age groups have increased so much, in fact, that a booster vaccine has been developed and is now highly recommended for adolescents 10–18 years of age. Thus, despite the availability of a vaccine, *B. pertussis* remains a significant pathogen that is responsible for an estimated 50 million cases of pertussis and 350,000 deaths worldwide.[35]

One of the problems associated with reducing the incidence of *B. pertussis*–related disease has been the lack of reliable culture methods that consistently allow for the isolation of *B. pertussis* from clinical samples. *B. pertussis* is a highly fastidious organism that requires special isolation conditions. Regan-Lowe or Bordet-Gengou media are specialized media that have been formulated to isolate *B. pertussis*. The media need to be inoculated with a freshly-collected nasopharyngeal swab and/or aspirate and incubated at 35°C in ambient air with increased humidity for at least 7–10 days in order to get the organisms to grow.[25]

B. pertussis very early became a target for the development of molecular-based assays because of its continued clinical significance and because of the difficulties encountered with the traditional culture methods used to isolate the organism. Once the *B. pertussis* genome was characterized, species-specific target sequences were identified and used to generate primers for PCR assays. Target sequences for *B. pertussis* are located in the IS*481* insertion sequence, the pertussis toxin gene promoter region, the adenylate cyclase gene, and species-specific porin protein structural genes.[25]

Several studies have been performed comparing the sensitivity and specificity of culture with PCR assays for the direct detection of *B. pertussis* in nasopharyngeal samples. Dragsted and colleagues found that the sensitivity of PCR for *B. pertussis* was 97%, whereas the sensitivity of the culture was only 58%. When culture as the gold standard was considered as 100% sensitive, they found that the PCR assay was 97% specific.[36] Fry, et al. found that using PCR to detect *B. pertussis* resulted in an almost fivefold increase in the ability to diagnose *B. pertussis* in clinical specimens as compared with culture.[37] In a third study comparing detection of *B. pertussis* in culture versus PCR, Chan and colleagues reported a sensitivity of 100%, specificity of 97.4%, positive predictive value of 87.6%, and a negative predictive value of 100% of PCR; for culture, they reported a sensitivity of 11.6%, specificity and positive predictive value of 100%, and a negative predictive value of 85.7%.[38] Another critical difference between culture and PCR that was investigated and reported by the Chan group was the amount of time needed to perform and report results from a PCR assay as compared with the amount of time required for *B. pertussis* to be detected in culture. They reported that results from PCR assays were available in 2.3 days (where the assay was only performed 3 to 5 days/week), whereas positive culture results were not available for 5.1 days (where cultures were performed and read 6 days/week).[38]

While molecular-based assays have clearly been shown repeatedly to have significantly higher sensitivity rates, some concerns still exist for the performance and interpretation of these assays. First of all, the major genetic target for primer binding in PCR assays detecting *B. pertussis* is IS*481*. This insertion sequence is also found in *Bordetella holmesii*, and thus the presence of *B. holmesii* in a clinical specimen can give false-positive results when *B. pertussis* is the target.[39] The amplification and detection of another insertion sequence, IS*1001*, can be used to discriminate between *B. pertussis* and *B. holmesii*. IS*1001* is used as a target to detect *Bordetella parapertussis* in clinical samples primarily, but IS*1001* is also found in *B. holmesii* and not in *B. pertussis*. IS*481* sequences are not seen in *B. parapertussis*. By amplifying both insertion sequence targets, the specificity of the assay for all three species is greatly increased.[40] Second, a standardized or FDA-approved, commercially available PCR test for *B. pertussis* is not yet available. A quality assurance program to assess interlaboratory performance of existing PCR assays for *B. pertussis* is also lacking at this time. Without a standardized assay or external assessment of internally developed assays, technologists in clinical laboratories must perform extensive validation studies and develop quality control and quality assurance programs to ensure the validity of in-house assays.

Nonetheless, PCR assays have completely replaced culture-based assays for the detection of *B. pertussis* in many laboratories, especially public health laboratories, and probably will eventually do so in all laboratories.

Chlamydophila (Chlamydia) pneumoniae

Chlamydophila (Chlamydia) pneumoniae is an obligate intracellular pathogen that causes 10% of community-acquired pneumonias and has recently been implicated in atherosclerosis and coronary artery disease. The prevalence of *C. pneumoniae* worldwide is high, with 70% of people having antibodies against *C. pneumoniae* by the time they are 50 years of age.[41] *C. pneumoniae* typically causes pharyngitis, bronchitis, and mild pneumonia. Analysis of chlamydial 16S and 23S rRNA sequences has led to the suggestion that the genus *Chlamydia* should be split such that *pneumoniae* is a species of a newly named genus, *Chlamydophila*.[42]

The traditional laboratory method used for the detection of *C. pneumoniae* in respiratory samples is culture of the organism on cell lines in a shell vial culture system that is used for viral detection. Culture, however, is insensitive and dependent on obtaining and inoculating fresh clinical samples containing infected host cells onto the cell lines. Because reliable laboratory methods for the detection of *C. pneumoniae* are lacking, many groups have tried to develop molecular-based assays targeting detection of *C. pneumoniae* in clinical samples. Unfortunately, the assays have thus far lacked the sensitivity and specificity necessary for utilization in routine clinical testing.[24,43]

Legionella pneumophila

L. pneumophila is the cause of Legionnaires' disease, a lower respiratory tract infection that was first diagnosed in men attending an American Legion convention in Philadelphia in 1976. Since their first identification, *Legionella* species have been found in water, both in the environment as well as in air conditioners and hot water tanks in a variety of types of buildings. *Legionella* species infections range from asymptomatic to fatal and are the third most common cause of community-acquired pneumonias.[44,45]

Laboratory diagnosis of *Legionella* includes culture of bronchoalveolar lavage (BAL) samples on buffered citrate yeast extract media (sensitivity = 80%; specificity = 100%), direct fluorescent antibody (DFA) stain (sensitivity = 33%–70%; specificity = 96%–99%), enzyme immunoassay for urinary antigen (sensitivity = 70%–80%; specificity = 99%), immunochromatographic assay for urinary antigen (sensitivity = 80%; specificity = 97%–100%), and serology (sensitivity = 40%–75%; specificity = 96%–99%), which is only useful retrospectively.[46] PCR assays are available for the direct detection of *Legionella* in respiratory tract specimens that are highly specific (88%–100% specificity), but they are not more sensitive than the traditional culture assays, with reported sensitivities ranging 64%–100%.[46] Thus far, primers for *Legionella* nucleic acid amplification have targeted the macrophage infectivity potentiator (*mip)* gene and 16S and 5S rRNA genes. Use of the 5S rRNA primers and early primers against 16S rRNA sequences has been associated with the low sensitivity and specificity that have been reported mostly because the primers did not amplify all of the clinically-relevant species of *Legionella*. Recent reports of PCR detection of *L. pneumophila* and *Legionella* species in BAL using different primers targeting 16S rRNA genes, however, did demonstrate amplification of multiple species of *Legionella*, but the assays are still in the developmental stages.[47, 48]

Mycoplasma pneumoniae

Mycoplasma pneumoniae is a *Mollicute,* a bacterium that lacks a cell wall and is the smallest in size and genome of the free-living organisms. *M. pneumoniae* is the most common cause of community-acquired pneumonia, causing 20% of these infections. Laboratory diagnosis of *M. pneumoniae* is accomplished primarily by culture of respiratory tract secretions on special media, but serological tests are also available. For culture, specimens must be inoculated into a mycoplasma transport medium, and *M. pneumoniae* grows very slowly, taking up to 4 weeks to be detected.

M. pneumoniae has been a target for the development of numerous molecular-based assays over the last 15 years.[24] Genes that have been the targets of amplification procedures include the P1 adhesion gene, 16S rRNA, the ATPase operon genes, and the gene (*tuf*) that encodes elongation factor 2. Amplification methodologies range from multiplex, nested, and real-time PCR to NASBA and Qβ-replicase. Primer design and amplification pro-

cedures for the nucleic acid of *M. pneumoniae* seem to be adequate and successful[18, 49-51]; however, problems have been encountered in evaluating the results of the amplification assays because when they are compared with isolation of *M. pneumoniae* in culture, the molecular-based methods detect many more positives than culture methods. When the nucleic acid amplification assays are compared with serological test results, the nucleic acid–based tests have a sensitivity ranging 77%–94% and a specificity ranging 97%–100%, depending on the study.[24] The real test of the sensitivity and specificity of the nucleic acid amplification assays is to compare the results to the isolation of *M. pneumoniae* in culture together with review of patient symptoms. PCR and culture results correlate better when the samples come from patients who have current lower respiratory tract infections than when the samples come from healthy individuals.[18] Respiratory secretions collected from healthy individuals that are positive in the PCR assay yet negative by culture suggest the persistence of the organism after an infection or asymptomatic carriage of the organism. Thus, just as with any other laboratory test, the results of nucleic acid amplification procedures need to be taken into consideration with patient symptoms, history, and other laboratory test results to ensure their validity in the diagnosis of the patient.

Despite the problems with the development, implementation, and interpretation of molecular-based assays for *M. pneumoniae*, *L. pneumophila*, *B. pertussis*, and *C. pneumoniae* individually, multiplex nucleic acid amplification tests that screen for the presence of all four organisms seem to show some promise in being able to detect these organisms in a sensitive, specific, and rapid manner.[53-55] As these organisms in particular have overlapping symptoms and traditional culture assays are difficult and/or time-consuming, multiplex assays that detect multiple respiratory tract pathogens will benefit physicians and patients in savings in time and cost yet provide sensitive and specific results leading to faster and more appropriate treatment and reduced hospital stays.[53,54]

Streptococcus pneumoniae

S. pneumoniae is the major cause of community-acquired pneumonia and is also a common cause of bacteremia, sepsis, otitis media, and meningitis.[25] Target age groups for infections causing disease are infants and toddlers younger than 3 years and adults older than 65 years. *S. pneumoniae* has been found in people 0-65 years of age colonizing the upper respiratory tract, although the highest rates of carriage are seen in children younger than 15 years of age. The virulence of *S. pneumoniae* in people at the extremes of age is due to the lack of an adequate immune response in these age groups since the production of antibodies that are critical for eradicating the organism is less than optimal in these groups because of the immaturity of the response in infants and the aging of the response in older adults.[25]

Traditional laboratory detection of *S. pneumoniae* is by culture of clinical samples. The organism is fastidious yet grows well on traditional media such as chocolate agar and trypticase soy agar with 5% sheep red blood cells. Because the organism can be found colonizing the oropharynx, the significance of its isolation in expectorated sputum that is contaminated with oral secretions is questionable. This fact makes the sensitivity of the culture of sputum for *S. pneumoniae* difficult to assess.

Molecular-based tests targeting *S. pneumoniae* have been in development for many years and have attempted to detect *S. pneumoniae* in a variety of clinical samples and by targeting a variety of genes (see Table 12.3). Unfortunately, the results have been mixed, and PCR is still not recommended as a method to diagnose *S. pneumoniae* infections.

In a study comparing the specificity of four different PCR assays for identifying *S. pneumoniae*, the authors found that primers specific for the autolysin (*lytA*) genes were the only primers that had 100% specificity for *S. pneumoniae*.[56] In another report, PCR assays targeting the pneumococcal pneumolysin gene were sensitive down to 10 colony forming units/mL, specific for *S. pneumoniae*, and determined to be positive on all blood (9) and cerebrospinal fluid (4) specimens that were culture-positive for *S. pneumoniae*.[57] The same assay performed on serum was positive in 38% of patients who had lobar pneumonia and in 44% of patients who had otitis media caused by *S. pneumoniae*. Unfortunately, the same PCR assay performed on serum was positive in 13%–30% of healthy children because of organism colonization of the upper respiratory tract.[57] On the other hand, PCR for *S. pneumoniae* on the serum of healthy adults age 18–50 years was negative in all of the subjects tested, suggesting that a positive PCR on an adult would

be a true positive result whereas on a child younger than 16 years might be a false positive due to upper respiratory tract colonization.

Another study of PCR targeting the pneumolysin gene performed on whole blood found that of the adults who had pneumonia caused by *S. pneumoniae*, the sensitivity of blood culture was 28%, pleural fluid culture was 60%, sputum culture was 20%, and PCR on blood was 55%.[58] Importantly in this study, PCR performed on blood of adults who had nonpneumococcal pneumonia was negative on all the patients, giving a specificity of 100%; 32% of patients in the study, however, who had pneumonia of unknown etiology had a positive PCR result for *S. pneumoniae*, and 4% of their control adults were also PCR-positive. Incorporation of these "false positives" reduced the specificity of the assay to 81%.

Even PCR assays developed to detect *S. pneumoniae* in respiratory tract samples have not shown the sensitivity and specificity necessary to supplant existing laboratory methods. Murdoch and colleagues published an evaluation of a PCR assay for *S. pneumoniae* and found that for sputum specimens in which *S. pneumoniae* was isolated in culture, 98% of the specimens were also positive by PCR.[59] They also performed PCR on throat swabs taken from patients with a clinical diagnosis of pneumonia, and 55% were positive for *S. pneumoniae* by PCR. As a control, they performed PCR on throat swabs of healthy people and found a similar rate of PCR positives (58%), further suggesting that although PCR is specific for *S. pneumoniae*, the clinical significance of a positive PCR assay is questionable because a significant portion of the population (especially children) is colonized with the organism and PCR can not discern between colonization and infection.[59]

Urogenital Tract Pathogens

Neisseria gonorrhoeae and *Chlamydia trachomatis* were among the first organisms to be targeted for detection in clinical specimens by molecular methods. The molecular methods are so well characterized for these two organisms that detection of the nucleic acid of *N. gonorrhoeae* and *C. trachomatis* is the laboratory method used almost exclusively. Other sexually transmitted bacteria are considered good targets for the development of molecular-based methods because traditional laboratory methods of detection and identification for these organisms either lack sensitivity or are time-consuming. Table 12.4 summarizes the molecular-based tests that have been described for the bacteria that cause genital tract infections.

Neisseria gonorrhoeae and Chlamydia trachomatis

N. gonorrhoeae and *C. trachomatis* are the two most common causes of sexually transmitted disease. Disease caused by *N. gonorrhoeae*, called gonorrhea, is associated with dysuria and urethral discharge in men and cervicovaginal discharge in women. *N. gonorrhoeae* can also cause pharyngitis and anorectal infections. *C. trachomatis* causes a nongonococcal urethritis and is asymptomatic in 50%–66% of men and women. *N. gonorrhoeae* and *C. trachomatis* are often found in coinfections, so it is prudent to rule out both organisms when considering that one is present.[61]

Traditional laboratory diagnosis of *N. gonorrhoeae* entails culture of endocervical or urethral swabs onto chocolate agar and selective, enriched media such as modified Thayer Martin. For male urethral swabs, Gram stain alone with the observation of gram-negative diplococci is diagnostic by itself for *N. gonorrhoeae* (sensitivity = 90%–95%; specificity = 95%–100%).[62] For female endocervical swabs or other specimen types from males and females, Gram stain alone is not diagnostic (sensitivity = 50%–70% for endocervical). *N. gonorrhoeae* is fastidious, and the specimen needs to be transported in a transport medium or plated directly onto media at the bedside. Delays in culturing the specimen are associated with false-negative cultures. Plates are examined daily for 72 hours for the presence of colonies resembling *Neisseria* and identified by biochemical testing.

Laboratory diagnosis of *C. trachomatis* is more problematic. *C. trachomatis* is an obligate intracellular pathogen; thus, when collecting specimens for isolation of *C. trachomatis*, it is critical that the practitioner scrape the endocervix or urethra to ensure the collection of host columnar epithelial cells that harbor the organisms. *C. trachomatis* is extremely labile, and clinical specimens sent to the laboratory for culture must be placed in a chlamydial transport medium to maintain the viability of the chlamydia. Culture of specimens for *C. trachomatis* is typically performed on McCoy cells in a shell vial system. The specimen is inoculated onto the cell line, the vial is incubated for 48–72 hours, and the cells are harvested and stained with a fluoresceinated antibody that has specificity for *C. trachomatis*. Cultures for *N. gonor-*

Table 12.4 **Typical Genital Tract Organisms Targeted by Molecular-Based Detection Methods**[25,60]

Organism	Specimen Sources	Traditional Diagnostic Methods	Gene Target
Treponema pallidum	Genital ulcers	Serological (indirect and direct)	TpN44.5a
	Blood	Direct antigen detection (dark field, DFA)	TpN19
	Brain tissue		TpN39
	Cerebrospinal fluid		p01A
	Amniotic fluid		TpN47
	Placenta		16S rRNA
	Umbilical cord		*polA*
	Fetal tissue		
	Serum		
Mycoplasma genitalium	Urine	Culture	MgPa (adhesion gene)
	Urethral		rDNA gene
	Vaginal		
	Cervical		
Mycoplasma hominis	Genital tract	Culture	16S rRNA
	Amniotic fluid		
Ureaplasma urealyticum	Genital tract	Culture	16S rRNA
	Amniotic fluid		Urease gene
Haemophilus ducreyi		Gram's stain	1.1 kb target
		Culture	*groEL* gene
		Serological	Intergenic spacer between 16S and 23S rDNA
			p27
			16S rDNA gene
Neisseria gonorrhoeae	Urine	Culture	*omp* III gene
	Urethral		*opa* gene
	Cervical		Cytosine DNA methyltransferase gene
	Thin preparation vials		*cPPB* gene
			Site-specific recombinase gene
Chlamydia trachomatis	Urine	Culture	MOMP
	Urethral	EIA	16S RNA
	Cervical	DFA	
	Thin preparation vials		
	Conjunctiva		

rhoeae and *C. trachomatis* have been considered the gold standard, but when compared with nucleic acid amplification assays, the sensitivity of culture for *N. gonorrhoeae* is 85%–100% and for *C. trachomatis* 80%.[9] Nucleic acid amplification assays have the additional advantages of being rapid, and testing can be batched and automated, resulting in further savings for the laboratory.

The first molecular-based assay available for *N. gonorrhoeae* and *C. trachomatis* was AccuProbe from GenProbe, Inc. The AccuProbe is a nonamplification-based nucleic acid hybridization method that detects the rRNA of an organism by using an acridinium-labeled single-stranded DNA probe. The sensitivity of the AccuProbe for *N. gonorrhoeae* as compared with culture is 100%, with a specificity of 99.5%. For *C. trachomatis*, the sensitivity of AccuProbe is 67%–96% (specimen quality is the reason for the variability), and specificity is 96%–100%.[9] Although the DNA probes have comparable sensitivities

and specificities to culture methods, laboratory professionals quickly implemented AccuProbe in their laboratories for the detection of *N. gonorrhoeae* and *C. trachomatis* because the assay is faster than culture and the same swab can be used for the detection of both *N. gonorrhoeae* and *C. trachomatis*.

Numerous nucleic acid amplification assays on the market target *N. gonorrhoeae* and *C. trachomatis*. The commercially available assays include target amplification assays such as COBAS AMPLICOR CT/NG (Roche Diagnostics, Indianapolis IN; PCR), BD ProbeTecET (Becton Dickinson Microbiology Systems, Sparks MD; strand displacement amplification), and APTIMA COMBO II (Gen-Probe Inc, San Diego CA; transcription mediated amplification). One signal amplification assay, called Rapid Capture System II for GC and CT (Digene, Gaithersburg MD; hybrid capture), is also available. The nucleic acid amplification assays can be performed on urethral or cervical swabs, urine, and, in some cases, on Thin prep transport vials that are used to collect cervical cells for Papanicolaou smears. The sensitivity of all of the amplification methods is excellent and ranges 93%–100%. Likewise, the specificity of these assays is excellent, ranging 99%–100%.[9] The ability to use urine as a specimen to screen for the presence of *N. gonorrhoeae* and *C. trachomatis* has many advantages. Urine is a noninvasive specimen that can be collected by the patient. The first portion of the urine stream should be collected for these assays from patients who have not voided for at least 2 hours. The acceptability of the Thin prep vials for *N. gonorrhoeae* and *C. trachomatis* testing means that one specimen can be collected from women for Papanicolaou smear, *N. gonorrhoeae*, *C. trachomatis*, and Human Papillomavirus.

In general, molecular-based assays are the major method for detection of *N. gonorrhoeae* and *C. trachomatis*. The assays are sensitive, specific, and rapid and have the potential to be fully automated. The only situation in which molecular-based tests is not acceptable for the detection of *N. gonorrhoeae* and *C. trachomatis* is in the workup of children in suspected child abuse cases. For these children, cultures should be performed alone or in conjunction with molecular-based assays. Another consideration for the use of molecular-based tests for *N. gonorrhoeae* and *C. trachomatis* is when laboratory testing has to demonstrate cure of an infection. As with any infectious organism and molecular-based tests, the nucleic acid is detectable in a clinical sample whether the organism is dead or alive. Another sample should not be taken for 3–4 weeks after treatment if the practitioner wants to see if the therapy was effective. Collection of samples and testing too soon after treatment will result in positives long after cultures on the same specimen have become negative.

Treponema pallidum

The spirochete *Treponema pallidum* subspecies *pallidum,* is the causative agent of syphilis, a sexually transmitted disease that results in the formation of a chancre at the site of inoculation (primary syphilis). If left untreated, the organism disseminates throughout the body, damaging tissues, and the patient may progress into the other stages of disease, i.e., secondary syphilis (disseminated rash), latent syphilis (asymptomatic period), and tertiary syphilis (central nervous system and cardiovascular manifestations).

Laboratory diagnosis of syphilis is limited to serological testing, in which patients are typically screened initially for the presence of antibodies against cardiolipin (a normal component of host membranes) (rapid plasma reagin [RPR] and venereal disease research laboratory [VDRL] tests) and followed up with testing for the presence of antibodies against *T. pallidum* (TP-PA test; Fujirebio) to confirm infection. *T. pallidum* cannot be grown in vitro. New enzyme immunoassay (EIA)–based tests are available that are being used to detect anti–*T. pallidum* antibodies. The EIA tests have been prepared using more immunologically relevant antigens and thus have consistently higher sensitivity (97%–100%) and specificity (98%–100%) for patients in all stages of syphilis. Laboratory professionals who have adopted the EIA assays use these assays to screen patients for syphilis and use the RPR to monitor effectiveness of treatment and diagnose reinfection.[63]

RPR and VDRL are limited in that, when reactive, they are not specific for syphilis, and even if the patient has syphilis, the sensitivity of the test in very early (77%–100%) and late (73%) syphilis is low.[63] The serological tests that detect *T. pallidum* antibodies are limited by the fact that they cannot differentiate between current and past infections: generally, once someone has syphilis, he or she will always have anti-*T. pallidum* antibodies. The RPR test, though, can be used to diagnose reinfections because titers of anticardiolipin antibodies will decrease to nonreactive following successful treatment of the organism and increase again with reinfections.

Several PCR assays have been developed and tested for the direct detection of *T. pallidum* DNA in genital ulcers, blood, brain tissue, cerebrospinal fluid, serum, and other samples with varying sensitivities (1–130 organisms).[60] Amplification of the *T. pallidum* DNA polymerase I gene (*polA*) resulted in a detection limit of about 10–25 organisms; when tested on genital ulcers, a sensitivity of 95.8% and a specificity of 95.7% were reported.[64] In another test of PCR for the detection of *T. pallidum* in anogenital or oral ulcers, the authors found that PCR was 94.7% sensitive and 98.6% specific; the positive predictive value was 94.7%, and the negative predictive value was 98.6% in patients who had primary syphilis.[65] The numbers were similar for patients in secondary syphilis, except that the sensitivity (80.0%) and positive predictive value (88.9%) were lower. Thus, while studies show some promise in the use of PCR for diagnosing syphilis, additional studies to test the sensitivity of PCR using clinical specimens still need to be performed in order for PCR to be routinely implemented in clinical laboratories for the detection of *T. pallidum*.[65]

Haemophilus ducreyi

H. ducreyi is a fastidious gram-negative coccobacillus that is the causative agent of chancroid. *H. ducreyi* is rarely found in the United States; it causes more infections in lower socioeconomic countries, especially in Africa, Asia, and Latin America. Laboratory diagnosis of *H. ducreyi* is difficult because the organism does not grow well in vitro and requires special media for isolation that is not available in most clinical laboratories in the United States. Gram stain of exudates is only 50% sensitive for *H. ducreyi* and thus is not recommended.[60]

PCR assays have been developed for *H. ducreyi* that amplify a variety of genes (see Table 12.4). Amplification of 16S rRNA along with the use of two probes for amplicon detection was shown by Chiu and colleagues to be 100% sensitive in detecting multiple strains of *H. ducreyi.*[66] In addition, the assay had a sensitivity of 83%–98% and a specificity of 51%–67% (depending on the number of amplification cycles) in detecting *H. ducreyi* in clinical specimens. Another group developed a PCR assay that amplified an intergenic spacer region between the *rrs* and *rrl* ribosomal RNA genes of *H. ducreyi* that was 96% sensitive for *H. ducreyi* in genital ulcer swabs compared with a sensitivity of 56% for culture.[67] Thus, PCR appears promising for the direct detection of *H. ducreyi* in genital specimens, but further development is needed before these assays are used routinely.

Mycoplasma and Ureaplasma spp.

Mycoplasma hominis, Mycoplasma genitalium, and *Ureaplasma urealyticum* cause nongonococcal urethritis. The mycoplasmas, as discussed above for *M. pneumoniae*, are the smallest free-living, self-replicating organisms known. *M. genitalium* has the smallest genome and thus was one of the first organisms to have its genome fully sequenced.[68] *M. genitalium* was first identified in 1981,[69] and culture methods first described in 1996[70] are still labor-intensive and not widely available. *U. urealyticum* is related to the *Mycoplasma* spp. and is also a member of the *Mollicutes* class.

PCR assays have been developed that amplify the adhesion gene (*MgPa*)[71,72] or the rDNA gene[73,74] of *M. genitalium*. Urethral or endocervical swabs or first-pass urine samples are all acceptable and yield positive PCR results. Although *M. genitalium* has been detected by PCR in all specimen types, its presence and association with disease are still questioned. Whereas more men who were symptomatic were positive by PCR for *M. genitalium* (20%), men who were asymptomatic still had detectable *M. genitalium* by PCR (9%).[71] Subsequent studies looking at *M. genitalium* by PCR in men with nongonococcal, nonchlamydial urethritis have shown that *M. genitalium* was the cause of symptoms in 18%–45.5% of cases.[75] Whereas PCR has been important in establishing *M. genitalium* as an important genital tract pathogen, the use of PCR as a clinical laboratory method for the routine diagnosis of *M. genitalium* has yet to be realized. Further studies are still required to validate the sensitivity and specificity of PCR for *M. genitalium* in genitourinary specimens.[60]

PCR assays for *M. hominis* and *U. urealyticum* have been developed but have not been used widely in clinical laboratories for the diagnosis of these organisms. The assays have been found to be specific and sensitive, but just like some of the organisms discussed above, without a reliable gold standard assay to use for comparison and especially in the absence of clinical symptoms, the clinical significance of PCR-positive specimens is difficult to interpret.[25]

Just like respiratory tract specimens, genital tract specimens have been the target for the development of multiplex assays in which the presence of nucleic acid of multiple organisms can be determined from one specimen

in one tube. The organisms causing genital tract infections either overlap in their symptoms, making diagnosis difficult without specific laboratory testing, or infections can be caused by the presence of multiple organisms at the same time. Several reports of multiplex PCR for genital tract specimens have been published. In one report, simultaneous detection of *T. pallidum*, *H. ducreyi,* and herpes simplex virus (HSV) types 1 and 2 in genital ulcers was performed by multiplex PCR.[76] The sensitivity of the PCR assay for HSV was 100% (culture sensitivity was 71.8%), for *H. ducreyi* was 98.4% (culture was 74.2%), and for *T. pallidum* was 91% (dark-field sensitivity was 81%). Since this first description of the multiplex assay, other groups have used the method to confirm the sensitivity of the assay for the targeted organisms and to use it to examine the prevalence of these organisms in various geographic areas in different years.[60]

Antimicrobial Agents

Antimicrobial agents are of two types, those that inhibit microbial growth (-**static**, e.g., bacteriostatic, fungistatic) and those that kill organisms outright (-**cidal**, e.g., bacteriocidal, fungicidal). Antimicrobial agents for use in clinical applications should be selective for the target organism with minimal effect on mammalian cells. The agent should also distribute well in the host and remain active for as long as possible (long half-life). Ideally the agents should have -cidal (rather than -static) activity against a broad spectrum of microorganisms.

Another way to classify antimicrobial agents is by their mode of action (Table 12.5). The ultimate effect of these agents is to inhibit essential functions in the target organism (Fig. 12-3). A third way to group antimicrobial agents is by their chemical structure. For example, there are two major types of agents that inhibit cell wall synthesis, the β-lactams with substituted ring structures and the glycopeptides.

Resistance to Antimicrobial Agents

Microorganisms naturally develop defenses to antimicrobial agents. Resistant *Staphylococcus, Pseudomonas,* and *Klebsiella* spp. are becoming commonplace in healthcare institutions. Long-term therapy with antibiotics such as vancomycin may lead to development of resistant clones of organisms. These clones may persist in low numbers below the detection levels of routine laboratory sensitivity testing methods.

There are several ways in which microorganisms develop resistance (Table 12.6). First of all, bacteria can produce enzymes that inactivate the agent. Examples of this resistance mechanism are seen in *S. aureus* and

Advanced Concepts

The first antibiotics isolated were natural secretions from fungi and other organisms. Synthetic modifications of these natural agents were designed to increase the spectrum of activity (ability to kill more organisms) and to overcome resistance. For example, cephalosporins include first-generation agents, cephalothin and cefazolin active against *Staphylococcus, Streptococcus,* and some Enterobacteriaceae. A second generation of cephalosporins, cefamondole, cefoxitin, and cefuroxime, is active against more Enterobacteriaceae and organisms resistant to β-lactam antibiotics. A third generation, cefotaxime, ceftriaxone, and ceftazidime, is active against *P. aeruginosa* as well as many Enterobacteriaceae and organisms resistant to β-lactam antibiotics. The fourth generation, cefepime, is active against an extended spectrum of organisms resistant to β-lactam antibiotics.

Table 12.5 **Mode of Action of Antimicrobial Agents**

Mode of Action	Examples
Disrupts cell wall synthesis or integrity	Beta-lactams (penicillins and cephalosporins)
	Glycopeptides (vancomycin)
Disrupts cell membrane structure or function	Polymyxins (polymyxin B)
	Bacitracin
Inhibits protein synthesis	Aminoglycosides (gentamicin)
	Tetracyclines
	Macrolides (erythromycin)
	Lincosamides (clindamycin)
Inhibits nucleic acid synthesis or integrity	Quinolones (ciprofloxacin)
	Metronidazole
Inhibits metabolite synthesis	Sulfamethoxazole
	Trimethoprim

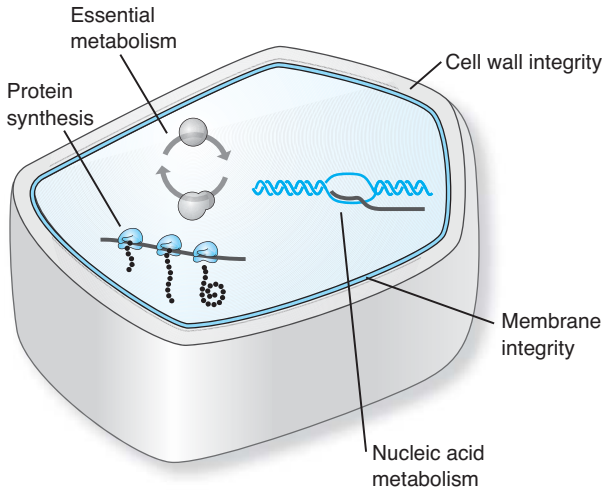


Figure 12-3 Sites of antimicrobial action. Depending on the type of organism, several structures can be affected by antimicrobial agents. All of these are essential for cell growth and survival.

N. gonorrhoeae that produce β-lactamase, an enzyme that cleaves the β-lactam ring of the β-lactam antimicrobials, such as the penicillins. Cleavage of the β-lactam ring destroys the activity of penicillin, rendering the organism resistant to the action of the penicillin. Second, organisms produce altered targets for the antimicrobial agent. Mutations in the gene encoding for a penicillin-binding protein, for example, change the structure of the protein such that penicillin can no longer bind to its target and thus loses its effectiveness. Finally, bacteria exhibit changes in the transport of the antimicrobial agent either into or out of the cell. An example of this mechanism is seen in gram-negative bacteria that change their outer membrane proteins (porins) in order to decrease the influx of the antimicrobial agent. If the agent cannot get into the cell and bind to its target, then it is not effective in inhibiting or killing the bacterium.

All these resistance mechanisms involve a genetic change in the microorganism (Table 12.7). These genetic changes are most commonly brought about by mutation and selection processes. If a mutation results in a survival or growth advantage, cells with the mutation will eventually take the place of those without the mutation, which are less able to survive and procreate. This process is stimulated by antibiotic exposure, especially if the levels of antibiotics are less than optimal. For example, *S. aureus* developed resistance to antibiotics that target its penicillin-binding protein (PBP1) by replacing PBP1 with PBP2a encoded by the *mec*A gene. PBP2a found in methicillin-resistant *S. aureus* (MRSA) has a low binding affinity for methicillin.

Another genetic resistance mechanism is the acquisition of genetic factors from other resistant organisms through transformation with plasmids carrying resistance genes or transduction with viruses carrying resistance genes. Genetic factors can also be transferred from one bacterium to another by conjugation (see Chapter 1 for a discussion of these processes). Genetically directed resistance can pass between organisms of different species. For example, MRSA (vancomycin-sensitive *S. aureus*) can gain vancomycin resistance from vancomycin-resistant *E. faecalis*. Vancomycin and other glycopeptides act by preventing the cross-linking of the peptidoglycan, thereby inhibiting cell wall production. Several genes have been found in enterococci that encode altered binding proteins,

Table 12.6 **Resistance Mechanisms**

Mechanism	Example	Examples of Agents Affected
Destruction of agent	β-lactamases	β-lactams
Elimination of agent	Multidrug efflux systems	β-lactams, fluoroquinolones, macrolides, chloramphenicol, trimethoprim
Altered cell wall structure	Thick cell walls that exclude agent	Vancomycin
	Altered agent binding sites	β-lactams
Alternate metabolic pathways	Altered enzymes	Sulfonamides, trimethoprim

Table 12.7 Genes Conferring Resistance to Antimicrobial Agents in Particular Organisms[78]

Organism	Antimicrobial Agent	Gene(s) conferring resistance
Staphylococcus aureus	Oxacillin	*mecA*
Streptococcus pneumoniae	Penicillin	*pbp1a* and *pbp1b*
Gram-negatives	β-lactams	*tem*, *shv*, *oxa*, *ctx-m*
Enterococcus	Vancomycin	*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*
Salmonella	Quinolones	*gyrA*, *gyrB*, *parC*, *parE*
Mycobacterium tuberculosis	Isoniazid Rifampin	*katG*, *inhA* *rpoB*

vanA, *vanB*, *vanC*, *vanD*, *vanE,* and *vanG*. The expression of *vanA* and *vanB* is inducible and transferred from cell to cell by plasmids carrying vancomycin resistance genes on a transposon[77] (Fig. 12-4). The resulting vancomycin-resistant *S. aureus* (VRSA) uses lactic acid instead of alanine to build its cell wall. The VRSA cell wall, then, does not contain the target structure (D-ala-D-ala) for vancomycin.

Mutations in the following genes are associated with the development of resistance to particular drugs: *rpoB* mutation is associated with rifampin resistance, and mutations in *katG*, *inhA*, *ahpC*, and *ndh* genes are associated with resistance to isoniazid.[78]

Molecular Detection of Resistance

Often the development of resistance is detected by performing in vitro **susceptibility testing**. Testing for altered sensitivity to antimicrobial agents is of clinical significance especially when organisms persist in patients being treated with antimicrobial agents that are generally considered effective against the particular isolate or when large numbers of organisms are observed in normally sterile fluids such as blood, cerebrospinal fluid, or urine. Susceptibility can be determined by phenotypic or genotypic methods. Phenotypic methods are generally used for aerobic bacteria, some mycobacteria, and yeast. For other organisms, such as viruses and filamentous fungi, phenotypic methods are not well standardized. Phenotypic methodologies include disk diffusion, broth dilution, and direct detection of resistance factors, such as β-lactamase.

Susceptibility testing measures the minimum inhibitory concentration (MIC) of an antimicrobial agent or the least amount of antimicrobial agent that is needed to inhibit the growth of an organism. There are established guidelines that state the MICs that are considered susceptible or resistant for a given organism and antimicrobial agent pair. Determination of MICs as a method to detect antimicrobial resistance is a phenotypic method. Although MIC methods are well established, and the results are generally reliable with regard to in vivo effectiveness of an agent for an organism, the methods can give equivocal results and are time-consuming, with results not available for at least 48 hours after the specimen is collected.

Molecular methods detecting genes directly involved in the resistance of an organism to a particular agent are being increasingly used in particular situations. There are four reasons for using molecular-based methodologies to determine antimicrobial resistance. First, when an organism has an MIC at or near the breakpoint of resistance, detection of mutated genes contributing to resistance would be irrefutable evidence not to use the agent. Second, genes involved in the resistance of organisms to antimicrobial agents can be detected directly in the clini-

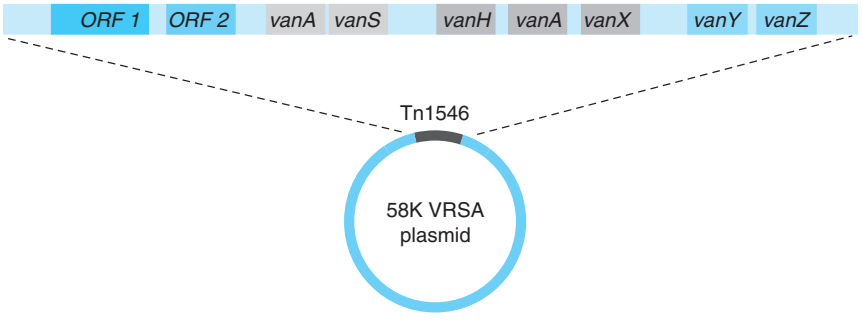


Figure 12-4 Vancomycin-resistant *S. aureus* (VRSA) plasmid carrying transposon Tn1546 with vancomycin-resistance genes.

cal specimen closer to the time of collection and save the time required to isolate the organism and perform phenotypic MIC determinations on isolated colonies. With no requirement for culturing potentially dangerous microorganisms, there is less hazardous exposure for the technologist as well. Third, monitoring the spread of a resistance gene in multiple isolates of the same organism is more useful in epidemiological investigations than following the trend in the MIC. Finally, molecular methods are considered the gold standard when new phenotypic assays are being developed.[78]

One of the most effective antibiotics, penicillin, was first used therapeutically in the early 1940s. Resistance to penicillin by the production of β-lactamases by organisms was first recorded soon after that. *Streptococcus pyogenes* is one of very few organisms that are still predictably susceptible to penicillin today. Penicillin and other β-lactam antimicrobials inhibit bacteria by interfering with an enzyme that is involved in the synthesis of the cell wall. In the laboratory, penicillin was modified to make it resistant to the β-lactamases (also known as penicillinases) that were being produced by the bacteria. Penicillinase-resistant penicillins, e.g., methicillin or oxacillin, were the products of that research. Staphylococcal infections were treated successfully with methicillin/oxacillin for years before the emergence of resistance was first observed in 1965.[79] MRSA and methicillin-resistant coagulase-negative staphylococci have become a major cause of infections acquired nosocomially as well as in the community. As described above, expression of an altered penicillin-binding protein (PBP2' or PBP 2a) encoded by the *mecA* gene is the mechanism by which these organisms have become resistant. Oxacillin cannot bind to the altered target, and therefore it has no effect on the bacterial cells.

Rapid identification of MRSA isolates in clinical specimens by direct detection of *mecA* is critical for effective patient management and prevention of nosocomial infections due to MRSA and has been accomplished through the development of PCR and other amplification assays that can be performed directly on a clinical sample. Many assays have been tested for sensitivity and specificity and have performed well.[79]

Enterococcus was the first organism in which glycopeptide resistance was observed.[80] Since then, vancomycin resistance has been observed in other organisms. Of most concern is emerging resistance to vancomycin in the staphylococci.[81] PCR was used to detect the resistance genes *vanA*, *vanB*, *vanC1*, and *vanC2* in fecal samples as a way to screen for vancomycin-resistant enterococci (VRE).[11] The specificity of the *vanA* primers was 99.6% when compared with isolation of VRE in culture. The use of four primers allowed for the detection of VRE in 85.1% of the samples. Real-time PCR has also been used to detect VRE in fecal surveillance specimens. Whereas PCR of *vanA* and *vanB* was more sensitive when performed on enrichment broths rather than directly from fecal swabs, 88% of specimens that were culture-positive for VRE were PCR-positive.[82] Because PCR is faster than traditional culture methods and has comparable sensitivity and specificity to culture, it is an attractive method for screening large numbers of samples for a particular target.

The use of molecular methods to detect antimicrobial resistance in *M. tuberculosis* is particularly attractive because traditional methods of determining antimicrobial susceptibility take days, if not weeks. The longer a patient with tuberculosis is inadequately treated, the more likely the organism develops resistance. Multidrug-resistant *M. tuberculosis* is a major problem around the world.[83] One group reported on the evolution of drug resistance of *M. tuberculosis* in a patient who was noncompliant with the treatment protocol. They found that in over 12 years of poorly treated tuberculosis, subpopulations of the organism emerged due to the acquisition and accumulation of mutations that rendered the organism resistant to isoniazid, rifampin, and streptomycin.[84] Many nucleic acid amplification protocols have been developed to directly detect mutations in the genes associated with conferring resistance to isoniazid and rifampin.[85–90] In general, these assays have demonstrated excellent sensitivity and specificity and provide rapid determination of drug susceptibility either directly from sputum or from cultures.

Thus, the advantages of using nucleic acid amplification assays for the determination of drug resistance is the rapid and specific detection of mutations in genes

Historical Highlights

Methicillin is no longer used for in vitro testing or in vivo therapy. The abbreviation MRSA is still used even though oxacillin or cefoxitin are used for in vitro testing and flucloxacillin and dicloxacillin are used in its place in vivo.

associated with resistance to particular antimicrobial agents, providing irrefutable evidence of resistance in a short period.

Molecular Epidemiology

An **epidemic** is a disease or condition that affects many unrelated individuals at the same time. A rapidly spreading outbreak of an infectious disease is an epidemic. A **pandemic** is a disease that sweeps across wide geographical areas. Epidemiology includes collection and analysis of environmental, microbiological, and clinical data. In microbiology, studies are performed to follow the spread of pathogenic organisms within the hospital (**nosocomial** infections), from the actions of the physician (**iatrogenic** infections), and in the community. Molecular epidemiology is the study of causative genetic and environmental factors at the molecular level. Results of epidemiological studies ascertain the origin, distribution, and best strategies for prevention of disease. In infectious disease, these efforts are facilitated by the ability to determine the genetic similarities and differences among microbiological isolates.

In the laboratory, molecular methods are very useful for identifying and typing infectious agents.[91] This is informative in a single patient for therapeutic efficacy as well as in groups of patients for infection control. Typing systems are based on the premise that clonally related isolates share molecular characteristics distinct from unrelated isolates. Molecular technology provides analytical alternatives from the chromosomal to the nucleotide sequence level. These genotypic methods, in addition to established phenotypic methods, enhance the capability to distinguish microorganisms. Whereas phenotypic methods are based on a range of biological characteristics, such as antigenic type or growth requirements, genotypic procedures target genomic or plasmid DNA (Table 12.8). Genome scanning methods, such as restriction enzyme analysis followed by PFGE, have been very useful in finding genetic similarities and differences. More recently, amplification and sequencing methods have been utilized for this purpose. The ability to discern genetic differences with increasing detail enhances the capability to type organisms regardless of their complexity. All methods, however, have benefits and limitations with regard to instrumentation, methodology, and interpretation.

Molecular methods are based on DNA sequence. DNA sequences range from highly conserved across species and genera to unique to each organism. Some of these sequences are strain- or species-specific and can be used for epidemiological analysis. DNA analysis is highly reproducible and, depending on the target sequences, can discriminate between even closely related organisms. Most (but not all) molecular methods offer definitive results in the form of DNA sequences or gel band and peak patterns that can be interpreted objectively, which is less difficult than subjective determinations that often require experienced judgment. With commercial systems, test performance has become relatively simple for some molecular epidemiology tests, whereas others require a higher level of laboratory expertise.

Table 12.8 Epidemiological Typing Methods

Class	Methods
Phenotypic	Biotyping, growth on selective media
	Antimicrobial susceptibility
	Serotyping, immunoblotting
	Bacteriophage typing
	Protein, enzyme typing by electrophoresis
Genotypic	Plasmid analysis
	Restriction endonuclease mapping
	Pulsed field gel electrophoresis
	Ribotyping
	Arbitrarily primed PCR, RAPD PCR
	Melt curve analysis
	REP-PCR, ERIC PCR, ITS, spa typing

Molecular Strain Typing Methods for Epidemiological Studies

In community or clinical settings, when the same organism is isolated multiple times, whether in the same patient or from different patients, the physician wants to know if the isolates were independently acquired, i.e., came from different sources, or if they came from the same source. With this knowledge, the physician can act to control the transmission of the organism, especially if it is being transmitted from a common source and that source has been identified. Most of the time, these analyses are performed on organisms that have been transmitted nosocomially, but sometimes procedures to determine relatedness are performed on isolates from community outbreak situations.[92]

There are many laboratory methods that can be used to determine the relatedness of multiple isolates, both phe-

notypic (e.g., by serology and antimicrobial susceptibility patterns) and genotypic (e.g., pulsed field gel electrophoresis and ribotyping).[93] The phenotypic methods suffer from a lack of reproducibility, and the ability to discriminate between isolates is not very good. Genotypic methods are used almost exclusively to type bacterial strains to determine the relatedness of multiple isolates and will be summarized in this section.

Plasmid Analysis

Plasmid analysis, or as it was known in the past, **plasmid fingerprinting,**[94] involves isolation and restriction mapping of bacterial plasmids. The same bacterial strain can have different plasmids carrying different phenotypes or resistance patterns. For this analysis, plasmid DNA is isolated from the specimen or culture and then digested with restriction enzymes. Plasmids are distinguished by the pattern of fragments generated when cut with the appropriate enzymes. Restriction analysis can also be performed on chromosomal DNA for organisms with small genomes. For organisms with larger genomes, whole genome restriction enzyme analysis often yields complex patterns that are more difficult to interpret.

Pulsed Field Gel Electrophoresis

Most molecular epidemiological tests are performed using **pulsed field gel electrophoresis** (PFGE), which can identify organisms with larger genomes or multiple chromosomes. For PFGE analysis, the DNA is digested with restriction enzymes that cut infrequently within the genomic sequences. The resulting large (hundreds of thousands of base pairs) fragments are resolved by PFGE (see Chapter 5 for a more detailed description of this system). Patterns of organisms will differ depending on the chromosomal DNA sequence of the organisms (Fig. 12-5). Tenover and colleagues devised a system to interpret the patterns of a test organism in comparison with the strain of organism known to be involved in the outbreak.[95] The interpretation of PFGE results is fairly straightforward and follows the "rule of three" (Table 12.9). This method has been used for typing numerous species, including strains of *Pseudomonas aeruginosa, Mycobacterium avium, Escherichia coli, N. gonorrhoeae*, VRE, and MRSA. Intralaboratory and interlaboratory computerized databases of band patterns can be stored for reference. A national PFGE database is stored at the Centers for Disease Control and Prevention (CDC) (www.cdc.gov/pulsenet/index.htm). One disadvantage to

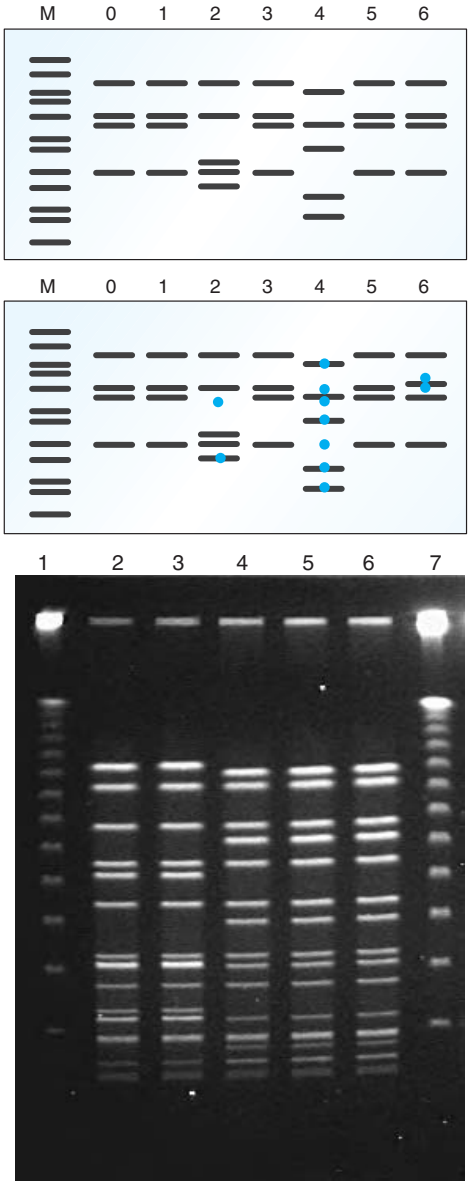


Figure 12-5 PFGE results of seven test strains (top panel). The fragment shifts are marked in the middle panel. Strains 1, 3, and 5 are the same as the outbreak strain (O). Strains 2 and 6 have apparently one genetic difference, resulting in two fragment differences. Strain 4 has seven fragment differences, indicating more than six genetic differences and is not related to the outbreak. PFGE of coagulase-negative *Staphylococcus* (bottom). Lanes 1 and 7, molecular weight markers; lanes 2 and 3 are two isolates that are same; lanes 4, 5, and 6 are three isolates that are the same but different from the isolates in lanes 2 and 3. (Courtesy of Mary Hayden, MD, Rush Medical Laboratories, Rush University Medical Center, Chicago, IL.)

Table 12.9 **Criteria for PFGE Pattern Interpretation**

Category	Genetic Differences*	Fragment Differences*	Epidemiological Interpretation
Indistinguishable	0	0	Test isolate is the same strain as the outbreak strain
Closely related	1	2–3	Test isolate is closely related to the outbreak strain
Possibly related	2	4–6	Test isolate is possibly related to the outbreak strain
Different	≥3	≥6	Test isolate unrelated to the outbreak

*Compared with the outbreak strain

performing PFGE to type strains is the time involved to perform the assay. It can take 2–3 days to complete one analysis.[91]

Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) analysis by Southern blot is the same technique first used to identify and investigate human genes (see Chapter 6). This method is performed by cutting DNA with restriction enzymes, resolving the resulting fragments by gel electrophoresis, and then transferring the separated fragments to a membrane for probing with a specific probe. Gene-specific probes are used to identify or subtype microorganisms such as *P. aeruginosa* in cystic fibrosis patients and nosocomial *L. pneumophila* infections.[96,97]

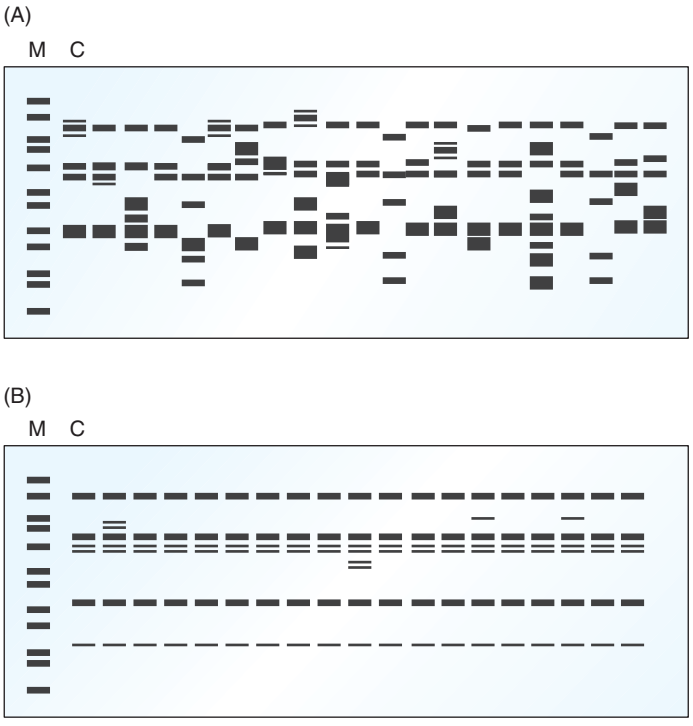
Insertion elements are segments of DNA that can move independently throughout the genome and insert themselves in multiple locations. Strains can be typed based on how many insertions are present and where they are located. Strains that are the same will have the same number and location of elements. For strain typing of *M. tuberculosis* by this method, the probe is complementary to IS*6110* and will bind to restriction fragments on the membrane that contain the insertion sequence, resulting in a series of bands that can be easily analyzed and compared.[98] This is the preferred method for typing *M. tuberculosis* isolates using the IS*6110* insertion element.[99]

The gene targets selected for this procedure depend on the organism under investigation and which genes will be most informative. Ribosomal RNA genes are highly informative over a range of microorganisms, which is the basis for a modification of the RFLP procedure called **ribotyping**. For this method, probes target the 16S and 23S rRNA genes. RFLP and ribotyping have been applied in industrial as well as clinical microbiology.[100,101]

RFLP can be investigated more rapidly using PCR amplification with gene-specific primers (**locus-specific RFLP**, or **PCR-RFLP**). This method requires amplification of specific regions by PCR (see Chapters 7 and 9 for more details on this PCR and PCR-RFLP). The amplicons are then cut with restriction enzymes, yielding bands of informative size. The advantage of this procedure, in addition to its speed, is the simple band patterns, which are much easier to interpret. Although the method is limited by the sequences that can be amplified and differentiated through restriction enzyme digestion, proper gene selection provides a highly reproducible and discriminatory test.[102] In one study, an 820-bp amplified fragment of the *ureC* gene from *Helicobacter pylori* digested with *Sau*3A and *Hha*I yielded 14 different *Sau*3A patterns and 15 different *Hha*I patterns. These patterns were informative as to antibiotic sensitivity of the various types to clarithromycin or clarithromycin-omeprazole dual therapy.[103]

Arbitrarily Primed PCR

Arbitrarily primed PCR, or random amplified polymorphic DNA (**RAPD**) assay, is a modified PCR using 10-base–long oligonucleotides of random sequences to prime DNA amplification all over the genome.[104,105] The gel pattern of amplicons produced is characteristic of a given organism. If two organisms have the same pattern, they are considered the same type. If the patterns differ, they are different types. The RAPD assay is relatively rapid and inexpensive; however, producing consistent results may be technically demanding. Accurate interpretation of RAPD raw data requires that the procedure con-



■ Figure 12-6 RAPD gel results. An unacceptable gel pattern is represented in panel A. The bands are smeared, and variable producing patterns are too complex for positive identification of unrelated strains. The gel pattern represented in panel B is acceptable. Strain differences can be clearly identified by variations from the known strain (C). Molecular weight markers are shown in lane M.

ditions are followed strictly so that pattern differences (not necessarily patterns) are reproducible (Fig. 12-6).

Amplified Fragment Length Polymorphism Assay

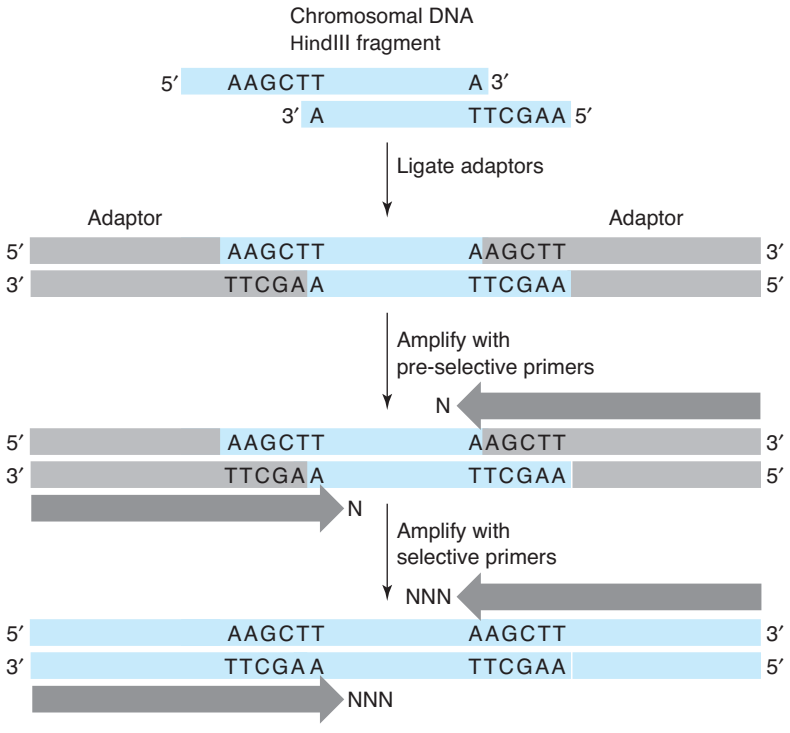
Amplified fragment length polymorphism (AFLP) is a name, rather than an acronym, chosen by the inventors for this assay due to its resemblance to RFLP.[106] The AFLP assay is based on the amplification of DNA fragments generated by cutting the test genome with restriction enzymes.[107] DNA isolated from the test strain is digested with *Hin*dIII or other restriction enzyme (Fig. 12-7). The adaptor-ligated fragments are then amplified in two steps with primers complementary to the adaptor sequences. Nucleotides placed on the 3' end of the primers select for specific sequences in the restriction fragments. The amplicons can then be resolved by gel or capillary electrophoresis (fluorescently labeled primers are used for capillary electrophoresis). The pattern will be characteristic of the strain or type of organism. This assay can be performed with one or two enzymes (e.g., *Eco*R1/*Mse*I or *Bam*H1/*Pst*I).

AFLP may detect more polymorphisms than RAPD analysis and is faster than PFGE. The procedure is more technically demanding, however, than REP-PCR. Gel patterns may also be complex (Fig. 12-8). Both high and low reproducibility of the method have been reported.[106,108]

Interspersed Repetitive Elements

Copies of conserved sequences are found throughout the genomes of most organisms. These sequences may have arisen from viral integration or movement of transposable elements (stretches of DNA that move from one location to another in a nonmendelian fashion, also called jumping genes). The genomic location of these structures are related to species type and can be used to distinguish between bacterial isolates.

Enterobacterial repetitive intergenic consensus (**ERIC**) sequences are 126-bp long genomic sequences found in some bacterial species that are highly conserved, even though they are not located in coding regions.[109] These sequences are located between genes in operons or upstream or downstream of single open reading frames.



■ Figure 12-7 AFLP analysis begins with restriction digestion of chromosomal DNA. The resulting fragments (top) are ligated with adaptors compatible with the restriction enzyme ends and complementary to primers used to amplify them. The first amplification is performed with preselective primers that end in a 3′ base (N) selected by the user. Selective primers with three added 3′ bases are used for a second round of PCR. This selection results in a characteristic pattern; only a fraction of the original fragments will be represented in the gel pattern.

ERIC sequences are flanked by inverted repeats that could form stem-loop or cruciform structures in DNA. These sequences are found only in gram-negative organisms, such as *Bartonella, Shigella, Pseudomonas, Salmonella*, *Enterobacter,* and others.

A related type of repetitive element, the repetitive extragenic palindromic (**REP**) sequence, is similar to the ERIC sequence in that it occurs in noncoding regions and contains an inverted repeat. REP sequences differ from ERIC sequences in size in being only 38 bp long, being more numerous in the genome, and being present in multiple copies at a single location (Fig. 12-9).[110] PCR primed from these elements yields a series of products that can be resolved by gel, capillary electrophoresis, or microfluidics[111] into characteristic patterns (Fig. 12-10). These elements have been used for typing of clinically important organisms such as *Clostridium difficile*[112] and fungal pathogens.[113]

Another repetitive element, **BOX,** was discovered in *S. pneumoniae.* BOX elements consist of different combinations of subunits, boxA, boxB, and boxC, 59, 45, and 50 bp long, respectively. Although these elements are not related to ERIC and REP sequences, they do form stem-loop structures, as do ERIC and REP. About 25 of these elements are present in the *S. pneumoniae* genome.

Internal Transcribed Spacer Elements

The ribosomal RNA genes comprise the most conserved region in the genome. These genes are arranged as an operon, including a small subunit, 18S rRNA, 5.8S rRNA, and a large subunit, 28S rRNA. The internal transcribed spacer (ITS) 1 and 2 elements (**ITS1** and **ITS2**) are found in regions separating the 18S and the 28S rRNA genes. ITS1 is located between the 18S and the 5.8S gene, and ITS2 is located between the 5.8S and the 28S rRNA genes. Two additional elements, intergenic spacer (IGS) regions, IGSI and IGSII, are located between the rDNA repeat units (Fig. 12-11). These elements are used for the identification and typing of yeast and molds. The ITS sequences are conserved within species but polymorphic between species.[114,115] The ITS can be amplified using primers directed to the unique 17S and 26S gene sequences. The resulting amplicons can be analyzed by sequencing, single-strand conformation polymorphism, density gradient gel electrophoresis, restriction enzyme analysis, or sequence-specific PCR.[116–118]

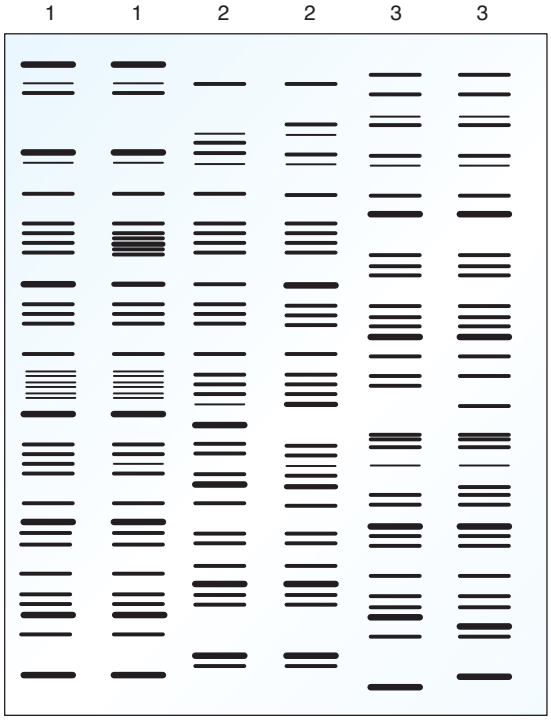


Figure 12-8 Banding patterns generated by fluorescent AFLP analysis. Note that duplicate specimens (1, 2, and 3) do not produce the exact pattern because of band shifts and different band intensities.

spa Typing

MRSA contains a variable number tandem repeat (VNTR) element in the 3′ coding region of the protein A gene (*spa*).[119] The element consists of repeat units 21 or 24 bp long. Repeat units also vary by sequence of at least one position, with 38 sequence types so far described. The *spa* element can have 2–16 repeat units. Analysis of these elements by PFGE or sequencing and comparison to known isolates (***spa* typing**) is used to identify MRSA. Almost 400 repeat profiles or *spa* types have been defined.

A similar sequence structure in the coagulase gene (*coa*) has also been used for *S. aureus* typing (***coa* typing**).[120] The *coa* VNTR units are 81 bp in length, and fewer than 70 repeat profiles have been described. PCR amplification and sequence analysis are used to analyze *coa* types. The discriminatory power of this method is increased by analysis of other repeated sequences elsewhere in the MRSA genome. The combination of *spa* and VNTR typing has discriminatory power equal to PFGE, with a more rapid turnaround time.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) is used to characterize bacterial isolates using sequences of internal fragments of housekeeping genes.[121,122] Six or seven genes are sequenced over 450–500 bases, and the sequences are assigned as alleles. Examples of housekeeping genes used in *S. aureus* MLST are shown in Table 12.10. Distinct alleles can be defined as a single base pair difference or multiple changes resulting from recombination or other genetic events. An isolate type is the collection of all seven alleles or the allelic profile, also called the sequence type. Bacteria have enough variation so that there are multiple alleles of each housekeeping gene (30 alleles/locus), making up billions of allelic profiles.[123,124] Because the data generated by MLST is text sequence, the results can be compared with those in large databases, e.g. pubmlst.org

Comparison of Typing Methods

Genotypic methods used for strain typing are evaluated and compared based on five criteria. First, the target organism must be typable by the method used to detect it

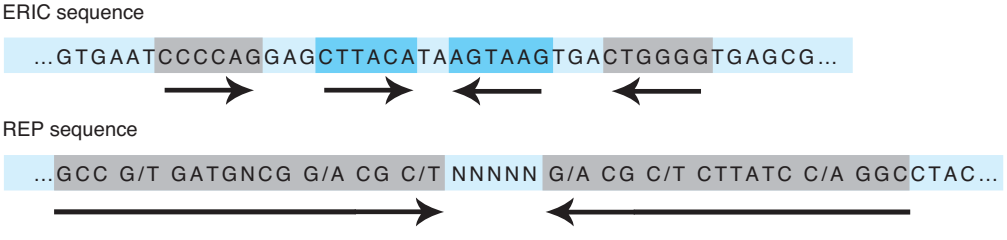


Figure 12-9 The central inverted repeat of an 126-bp ERIC sequence (top) and a consensus REP sequence (bottom). ERIC sequences contain multiple inverted repeats (arrows) that can generate a secondary structure consisting of several stems and loops. REP sequences contain a conserved inverted repeat that forms a stem with a loop that includes 5 bp of variable sequence (N).

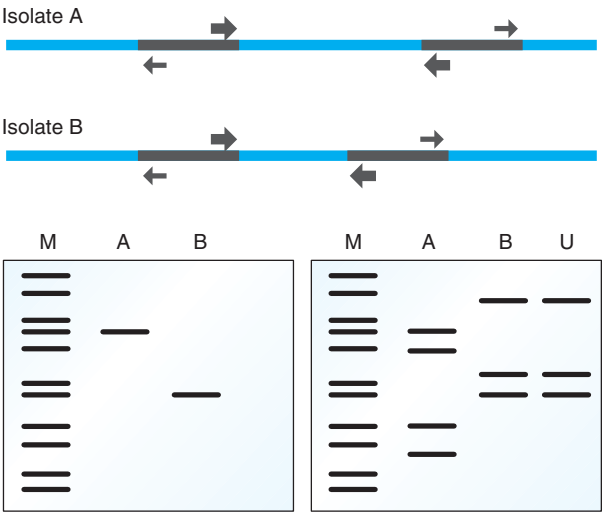


Figure 12-10 Species identification by REP or ERIC primed amplification. REP and ERIC sequences are present in different chromosomal locations in bacterial subtypes A and B. PCR, which extends outward oriented primers hybridized to the repeated sequences, generates amplicons of different sizes based on the placement of the element as shown in the gel depicted in the bottom left panel where only one amplicon is shown. Multiple REPs and ERICs throughout the bacterial chromosome generate multiple amplicons with characteristic gel patterns (bottom right). An unknown isolate (U) is identified as the same strain as isolate B. M, molecular weight markers

(**typing capacity**). A test that detects a genotypic or phenotypic characteristic that is not expressed in all members of a species will not accurately detect the target organisms at all times. A molecular assay must target a reasonably polymorphic DNA sequence, alleles of which are unambiguously associated with a given strain. Second, the method must be **reproducible**. A reproducible method yields the same result on repeated testing of the same organism or strain. Variations in cell characteristics, such as antigens or receptor expression, lower reproducibility (precision). Third, the method must clearly distinguish between unrelated strains (**discriminatory power**). Reproducibility and discriminatory power are important for establishment of databases that can be used by independent laboratories. Fourth, **ease of interpretation** of results is important. Unclear or complex results will lower reproducibility and discriminatory power. Finally, **ease of test performance** is important in order to minimize chance of error or ambiguous results. Rating of representative methods is shown in Table 12.11.

Table 12.10 **Example of Housekeeping Genes Sequenced in an MLST Test**

Gene	Gene Product	Number of Alleles
ArcC	Carbamate kinase	52
AroE	Shikimate dehydrogenase	88
GlpF	Glycerol kinase	55
Gmk	Guanylate kinase	51
Pta	Phosphate acetyltransferase	57
tpi	Triosephosphate isomerase	74
YqiL	Acetyl coenzyme A acetyltransferase	66

The most desirable typing method is the one that will type all strains and have excellent reproducibility, discriminatory power, and ease of performance and interpretation. Unfortunately, no such method fits this profile, so the laboratory professionals performing these types of analyses may have to sacrifice ease of performance, for example, in order to get excellent discriminatory power when they are choosing which molecular typing method will be performed in their laboratory.

In conclusion, molecular-based methods are available for the detection, identification, and characterization of a number of bacteria. For some organisms, the assays are used almost exclusively, such as for *N. gonorrhoeae*, *C. trachomatis*, and *B. pertussis*. For other organisms, molecular-based tests are used to provide rapid results and supplement traditional testing such as for *M. tuberculosis*. Finally, molecular-based methods are still in the

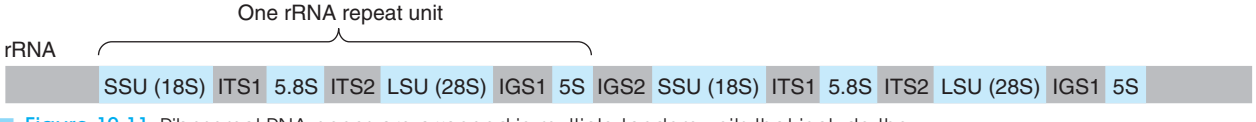


Figure 12-11 Ribosomal RNA genes are arranged in multiple tandem units that include the major rRNA transcript (18S-28S) and the 5S gene. ITSs are located within the major transcript template area and IGSs in the region between the repeat units surrounding the 5S rRNA gene.

Table 12.11 **Performance Comparison of Representative Molecular Epidemiology Methods***

Method	Typing Capacity	Discriminatory Power	Reproducibility†	Ease of Use	Ease of Interpretation‡
Plasmid analysis	Good	Good	Good	High	Good
PFGE	High	High	High	Moderate	Good–moderate
Chromosomal RFLP	High	Good	Good	High	Moderate–poor
Ribotyping	High	High	High	Good	High
PCR-RFLP	Good	Moderate	Good	High	High
RAPD	High	High	Poor	High	Good-High
AFLP	High	High	Good	Moderate	High
Repetitive elements	Good	Good	High	High	High
Sequencing	High	High	High	Moderate	Good-High

*from Olive and Bean[89]
†Intralaboratory
‡Interpretation is influenced by the quality of the data

development stage for most bacteria and will probably be performed more routinely in the future.

Viruses

Molecular-based methods have benefited the laboratory diagnosis of viruses probably more than any other organism. In general, viruses are diagnosed by testing for antibodies against the virus, by measuring the presence or absence of viral antigens, or by detecting the growth of a virus in a culture system. Although some of these methods are well-established for certain viruses, they all have major disadvantages associated with them.

Even though laboratory testing is available for antibodies against most viruses, the detection of antibodies against a virus is an indirect method of diagnosis. The host immune response needs to be stimulated by the virus to produce antibodies. If a patient is immunodeficient and does not make antibodies, the lack of antibodies is due to host factors and not due to the lack of the virus, although the lack of antibodies is often interpreted as a lack of the virus. Using antibodies to diagnose an infection is often a retrospective indication of the infection. To interpret antibody testing with the most confidence, paired sera should be collected, one collected during the acute phase of the infection and the other collected as the patient is convalescing, and the titers of antibodies measured in both samples. A fourfold or greater rise in titer level from the acute sample to the convalescent sample indicates the presence of the virus during the acute stage. Detecting IgM antibodies in particular during an acute infection is the best evidence for the presence of that virus. But even detecting IgM, the first isotype of antibody produced in an acute infection, is not without problems. If the patient is in the very early stages of infection, IgM titers may be below detection limits and would be interpreted as negative. When the patient is infected with a virus and the antibodies are not detectable, they are in the "window" period. During this time, the patient is infected and infectious, yet antibodies are not detectable.

Antigen detection testing is available in the clinical laboratory for only some viruses. Assays that measure viral antigens are available more often for Respiratory Syncytial Virus, Influenza Virus, Rotavirus, Herpes Simplex Virus (HSV), Cytomegalovirus (CMV), and Hepatitis B Virus (HBV). Viral antigens are detected by enzyme immunoassays or direct immunofluorescent assays most often.

The primary method used in clinical virology laboratories to detect and identify viruses in body fluids is by tissue or cell cultures. Monolayers of host cells are grown in vitro, the patient's specimen is inoculated onto the cells, and changes in the cells due to viral infection, called cytopathic effect, are observed microscopically by the technologist. The identity of the virus is confirmed using fluorescently labeled monoclonal antibodies. While culture is often the gold standard for many viruses, in particular, Adenovirus, Enteroviruses, CMV, Influenza, and HSV, it is not applicable for other viruses because the viruses do not grow in current in vitro culture systems, such as the hepatitis viruses. Another disadvantage to viral culture is the amount of time before viral growth is

detectable. Although centrifuging the sample onto the monolayer in the shell vial system has decreased detection time; days to weeks, depending on the virus, can pass before detection of cytopathic effect. Furthermore, some viruses do not produce a cytopathic effect on infecting cells, or the cytopathic effect that is produced is subtle and easily missed. In these cases, the cultures will be reported as a false negative. Another disadvantage of using viral cultures to detect and identify viral infections is that the specimen must be collected in the acute phase of the disease, i.e., in the first 5 days of the illness. After 5 days, the amount of virus in body fluids decreases significantly and may result in false-negative cultures.

As current diagnostic methods used to detect and identify viruses have so many disadvantages, tremendous opportunity exists to fill that void with molecular-based assays. Nucleic acid amplification assays have quickly become indispensable in the clinical virology laboratory. Molecular methods are well suited to target the various configurations of nucleic acids found in human pathogenic viruses (Table 12.12). Target amplification assays such as PCR, reverse transcriptase PCR (RT-PCR), quantitative (or real-time) PCR (qPCR), and transcription-mediated amplification (TMA) as well as signal amplification assays such as branched DNA (bDNA) amplification and hybrid capture are used in the clinical virology laboratory to diagnose or monitor viral infections. Table 12.13 is a summary of the viruses for which nucleic acid amplification assays are available (either commercially or performed in-house) along with the type of amplification procedure, the targeted genes, and clinical utility. Molecular-based tests for HIV and HCV are used more extensively in clinical laboratories; they are discussed in more detail below.

Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is unique among the RNA viruses in that it is a retrovirus that makes a DNA copy of genomic RNA using virally encoded reverse transcriptase. There are two types of HIV: HIV-1 and HIV-2. HIV-2 is a minor isolate found mainly in West Africa and is less pathogenic than HIV-1, causing more latent infections and, rarely, AIDS. HIV rapidly mutates and recombines so that there are multiple groups that are then divided into "clades," or subtypes, of the virus that are found in different places of the world.[25,128] HIV group

Table 12.12 Genomes of Human Viruses

Double-Stranded DNA Viruses
Adenovirus
BK virus
Cytomegalovirus
Epstein-Barr virus
Hepatitis B virus
Herpes simplex virus 1
Herpes simplex virus 2
Human papillomavirus
JC virus
Molluscum virus
Rotavirus
Vaccinia virus
Varicella-zoster virus
Single-stranded DNA virus:
Parvovirus
Double-stranded RNA virus:
Rotavirus
Single-stranded RNA viruses:
Arboviral encephalitis viruses*
Colorado tick fever virus
Coronavirus*
Coxsackie virus*
Dengue virus*
Echovirus*
Hepatitis A virus*
Hepatitis C virus
Hepatitis D virus
Hepatitis E virus
Human T-cell leukemia virus‡
Human immunodeficiency virus‡
Influenza virus
Measles virus
Mumps virus
Norwalk virus, norovirus
Parainfluenza virus
Poliovirus*
Rabies virus
Respiratory syncytial virus
Rhinovirus*
Rubella virus*
Yellow fever virus*

*Positive RNA; directly translated
†Negative RNA; complimentary to the translated strand
‡Retroviral replication requires a DNA intermediate

Table 12.13 **Nucleic Acid Amplification Tests for Viruses** [8,125–127]

Virus	NAA Methodology	Amplified Target	Dynamic Range/Sensitivity	Clinical Utility
Human immunodeficiency virus	PCR: HIV-1 DNA (Roche)	*gag* gene; 155 bp (HIV-1 group M (subtypes A-H), not HIV-2 or HIV-1 group O)	400-750,000 copies/mL (standard) 50-100,000 copies/mL (ultrasensitive)	Viral quantitation Disease prognosis Treatment monitoring
	RT-PCR (Amplicor and COBAS; Roche)			
	NASBA: NucliSense (bio Merieux)	*gag* (similar to Amplicor) HIV-1 groups M, O, and N	176,000–3,470,000 copies/mL	
	bDNA: Versant HIV-1 (Bayer)	*pol*; subtypes of group M (subtypes A-G), but not group O	75–500,000 copies/mL	
Cytomegalovirus (CMV)	Hybrid capture (Digene)	Immediate-early antigen 1	1400-600,000 copies/ml	Detect CMV DNA in organ transplant and AIDS patients and congenitally infected infants
	PCR	Major immediate-early antigen	400-50,000 copies/mL	
	NASBA	Glycoproteins B and H		Viral load determinations
	qPCR	Major capsid protein		Detect HSV when asymptomatic or when cultures are negative
Herpes simplex virus-1 and -2 (HSV)	PCR	Thymidine kinase		
	qPCR	DNA polymerase DNA-binding protein Glycoproteins B, C, D, and G		Diagnosis of HSV encephalitis and neonatal infections
Epstein-Barr virus (EBV)	PCR	EBNA1		EBV-associated malignancies
	qPCR	LMP-1		Detect EBV in asymptomatic immunocompromzised hosts
Human papillomavirus (HPV)	Hybrid capture (FDA-approved)	L1 or E1 open reading frames	10^5 copies/mL	Detection of HPV in endocervical swabs
	PCR			Differentiation of low-risk and high-risk types
	qPCR			Monitoring women with abnormal Pap smears
Hepatitis B virus	PCR		1000–40,000 copies/mL	Prognosis and monitoring of antiviral treatment response
	bDNA		0.7–5000 meq/mL	
	Hybrid capture		142,000–1,700,000,000 copies/mL (standard) 4700–56,000,000 copies/mL (ultrasensitive)	
Parvovirus B19	PCR			Diagnosis of infections

Continued on following page

Table 12.13 **Nucleic Acid Amplification Tests for Viruses** [8,125–127] *(continued)*

Virus	NAA Methodology	Amplified Target	Dynamic Range/Sensitivity	Clinical Utility
Respiratory syncytial virus (RSV)	RT-PCR	Fusion glycoprotein (F) gene Nucleoprotein (N) gene		Detection of RSV Differentiate between subgroups A and B
Parainfluenza viruses	RT-PCR	Hemagglutinin-neuraminidase conserved regions 5′ noncoding region of F gene		Epidemiology
Influenza viruses	RT-PCR	Conserved matrix (M) genes (influenza A and B) Nucleocapsid protein (influenza A) NS1 gene (influenza B)		Diagnose infections Characterize isolates Can be type- and subtype-specific
Metapneumovirus	RT-PCR	Fusion (F) gene RNA polymerase (L) gene		
Coronavirus	RT-PCR	RNA polymerase gene Nucleoprotein gene		Used to detect and characterize the SARS virus
Norwalk virus	RT-PCR	RNA polymerase gene		
Rotavirus	RT-PCR	VP7 gene VP4 gene		
Hepatitis C virus	RT-PCR bDNA	5′ untranslated region (UTR) 5′ UTR and core protein gene	600-800,000 IU/mL 3200-40,000,000 copies/mL	
West Nile virus	RT-PCR NASBA	Variety of gene targets based on genome of type strain NY99	0.1–1 PFU 0.01 PFU	Used for diagnosis and surveillance
Rubella virus	RT-PCR	Surface glycoprotein, E1, gene	Variable; Sensitivity of 3–10 copies is best	Primarily for fetal diagnosis Used for diagnosis when serum is not available May be used to confirm positive serological results
Mumps virus	RT-PCR	Hemagglutinin, neuraminidase, P, SH, and F genes		Differentiate strains
Measles virus	RT-PCR	M, H, F, N		When culture is not practical or genotyping is required for diagnosis of MIBE or SSPE Differentiation of vaccine and wild-type strains

Continued on following page

Table 12.13 **Nucleic Acid Amplification Tests for Viruses** [8,125–127] *(continued)*

Virus	NAA Methodology	Amplified Target	Dynamic Range/Sensitivity	Clinical Utility
Enteroviruses (group A and B Coxsackieviruses, echoviruses, and others)	RT-PCR	Conserved 5′ nontranslated region		Performed on CSF to rule out enteroviral meningitis
BK Virus (polyomavirus)	PCR	Large T protein		Diagnosis of progressive multifocal leukoencephalopathy Detection in kidney transplant patients

MIBE = Measles inclusion body encephalitis
SSPE = Subacute sclerosing panencephalitis
PFU = Plaque-forming units

M causes 95% of the infections due to HIV around the world. Group M is further divided into eight clades (A, B, C, D, F, G, H, and J). Group M, clade B, is found most often in the United States and Europe. Group O HIV is found primarily in West Africa, and group N is found in Cameroon.[128]

To infect host cells, the HIV surface molecules gp120 and gp 41 interact with CD4, a molecule that is expressed primarily on the surface of helper T lymphocytes and is also found on macrophages, dendritic cells, and other antigen-presenting cells. Chemokine receptors, in particular CCR5, on dendritic/Langerhans' cells and macrophages/monocytes, and CXCR4 on CD4+ T cells form a complex with CD4 on the cell surface and also engage gp120. After attachment to host cells via CD4-gp120 binding, the virus enters the cell, where reverse transcriptase makes cDNA from viral RNA. The cDNA integrates into the host DNA where it either persists in a stage of latency as the provirus or is replicated actively. Transcription and translation of viral peptides as well as production of viral RNA are performed by cellular components under the direction of virally encoded regulatory proteins (i.e., *tat*, *rev*, *nef*, and *vpr*).[128]

Diagnosis of HIV infection is accomplished by detecting antibodies specific for HIV in an EIA and confirming the specificity of detected antibodies for HIV products in a Western blot. In infants who have maternal IgG and in patients suspected of incubating HIV in whom antibody tests are negative, antigen detection tests are available that measure the amount of HIV p24 antigen. Nucleic acid amplification assays are performed after someone has been diagnosed as having HIV to determine how actively the virus is replicating (viral load), when to start antiretroviral therapy, and to monitor efficacy of treatment.

Viral Load Determination

The amount of HIV or viral load is used as a marker for disease prognosis as well as to track the timing and efficacy of antiretroviral therapy. Patients are 10 times more likely to progress to AIDS within 5 years if they have viral loads above 100,000 copies/mL within 6 months of seroconversion.[128] The aim of antiretroviral therapy is to get the viral load below 50 copies/mL of blood. Patients who maintain viral levels at less than 10,000 copies/mL in the early stages of the infection are at decreased risk of progression to AIDS.

Patients who are effectively treated with antiretroviral therapy will have a significant reduction in viral load 1 week after the initiation of therapy. The lack of significant decrease in viral load during this time indicates the lack of efficacy.[128] Highly active antiretroviral therapy (HAART), consisting of two reverse transcriptase inhibitors combined with a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor, have been shown to reduce viral loads below the detection limits of even ultrasensitive assays; these levels remain undetectable for more than 3 years.[129] In patients receiving HAART, 2 $\log_{10}$ decreases in viral load have been documented. Viral load

testing should be performed in conjunction with the determination of CD4 counts. In general, but not always, viral load and CD4 counts are inversely proportional; that is, the higher the viral load, the lower the CD4 count.[127]

Molecular-based methods that are used to quantify HIV in patient samples include PCR for integrated DNA, RT-PCR for viral RNA, nucleic acid sequence-based amplification (NASBA), and bDNA (see Table 12.13). See Table 12.14 for a comparison of the advantages and disadvantages of each of these assays for HIV viral load determination.[127] Viral load determinations should be made before therapy is started, 2–8 weeks after therapy initiation to see the initial response, and then every 3–4 months to assess therapeutic effectiveness. Because nucleic acid amplification assays use different amplification techniques, and sometimes different standards for quantifying, results from one method may not be comparable with another method. In general, viral loads determined by the Amplicor RT-PCR (version 1.0) are about twofold higher than loads determined either by bDNA (version 2.0) or by NASBA.[130,131] On the other hand, viral loads determined by the bDNA (version 3.0) and Amplicor RT-PCR (version 1.5) were similar.[132] Even though results obtained by different methods are becoming more and more comparable, it is still recommended that laboratory professionals use the same method to determine viral loads when monitoring patients over time.

Quantitative HIV-1 RNA testing in plasma has been the standard for monitoring drug therapy and HIV disease progression. Laboratory measurements of viral load and clinical disease have been described by the Multicenter AIDS Cohort Study, an ongoing project monitoring the clinical histories of treated and untreated HIV-infected men. The Department of Health and Human Services (DHHS) recommends an objective of maximal suppression of viral replication down to undetectable levels by sensitive analysis.

The first commercially produced tests could detect viral loads down to 400–500 copies/mL plasma; however, suppression to less than 20 copies/mL plasma was associated with longer response to therapy than suppression below 500 copies/mL.[133] These observations emphasize the importance of a highly sensitive assay with a very low limit of detection for optimal treatment strategy and patient care. The lower **limit of detection** is defined as the lowest viral concentration that can be detected 95% of the time in the test assay. Currently available FDA-approved HIV tests include the Bayer VERSANT HIV-1 RNA 3.0 Assay (bDNA), the Roche Amplicor MONITOR Test (RTPCR), and the bioMerieux NucliSens HIV-1 QT Test (NASBA), with lower limits of detection of 75, 50, and 176 viral copies per ml plasma, respectively. The Abbott RealTime™ HIV-1 (qPCR) assay has also been developed as a more automated method. This test has a limit of detection of 40 viral copies/mL.

In addition to sensitivity, all test methods including HIV tests should have certain characteristics (Table 12.15). Accuracy is an important feature of viral load testing. Accuracy is established by calibration of assays to a common standard. The test must have quantitative as well as qualitative accuracy that is a true measure of the viral level over a range of values. DHHS and the International

Table 12.14 Advantages and Disadvantages of the Nucleic Acid Amplification Methods for HIV Viral Loads

Test	Advantages	Disadvantages
bDNA	High throughput Broad dynamic range Applicable for group M subtypes A–G	No internal control False positives reported
Amplicor RT-PCR	Internal control Good specificity	Limited dynamic range
NASBA	Broad dynamic range Performed on many specimen types and volumes	Does not detect all non-B subtypes

Table 12.15 Test Performance Features for Viral Load Measurement

Characteristic	Description
Sensitivity	Lowest level detected at least 95% of the time
Accuracy	Ability to determine true value
Precision	Reproducibility of independently determined test results
Specificity	Negative samples are always negative and positive results are true positives
Linearity	A serial dilution of standard curve closely approximates a straight line
Flexibility	Accuracy of measurement of virus regardless of sequence variations

AIDS Society have set viral load levels for initiation of therapy. These levels must be consistently identified in independent laboratories as accurately as possible. Real-time PCR methods offer linear measurement over a wider range than other methods. This precludes the requirement for dilution of high-titer specimens before analysis. The precision or reproducibility of the test is important for establishment of statistically significant differences in the viral load over a serial testing period. The DHHS defines a minimally significant change as a threefold increase or decrease in viral load/mL plasma. High specificity of a test provides confidence that a positive result is truly positive. All subtypes of virus should be detected with equal efficiency to avoid under- or overestimating viral loads of certain subgroups.

There is not much variability in viral loads among repeated samples for all of the methods, e.g., quantifying RNA in the same patient will not change much (approximately 0.3 $\log_{10}$ unit) over time as long as the patient is clinically stable and antiretroviral therapy has not begun or changed.[134] In order to be clinically relevant, viral load changes from one determination to another must be at least threefold different (0.5 $\log_{10}$ unit). HIV-positive patients may experience transient increases in viral loads when they have other infections or receive vaccinations, but levels will return to baseline within a month.[135-137]

Proficiency testing is available from the College of American Pathologists (CAP; Northfield, IL) and the CDC. The World Health Organization (WHO) has an HIV-1 RNA reference standard. Acrometrix (Benicia, CA), Boston Biomedica, Inc. (West Bridgewater, MA), and IMPATH-BCP, Inc. (Franklin, MA) have external standards and verification panels for HIV-1 viral load testing.

Genotyping

Genotyping is used to monitor the development of antiretroviral drug resistance in HIV. Most of the antiretroviral drugs target the reverse transcriptase and protease enzymes, so these are the genes that are most often examined in genotyping procedures. Thus far, more than 100 mutations associated with drug resistance have been identified in HIV-1.[138,139]

To perform genotyping, viral RNA is extracted, and PCR is used to amplify the whole protease gene and part of the reverse transcriptase gene. The products are analyzed for the presence of mutations by sequencing, hybridizing onto high-density microarrays (GeneChip, Affymetrix, Santa Clara, CA), or reverse hybridizing using the line probe assay (VERSANT HIV-1 Protease and RT Resistance Assays, LiPA, Bayer Diagnostics). Sequencing is performed most often; there are currently two commercially-available kits for this purpose: Trugene HIV-I genotyping kit and OpenGene DNA-sequencing system (Bayer Corp., Tarrytown, NY) and ViroSeq HIV-1 genotyping system (Applied Biosystems, Foster City, CA). Once the sequence of the patient's virus is known, it must be compared with a wild-type sequence to identify the mutations present in the patient's isolate if any.

After the mutations have been identified, the significance of those mutations for their impact on antiretroviral therapy must be assessed, and this is generally accomplished through the analysis of mutations by computer algorithms. Interpretation of genotyping results with regard to the impact of a mutation on the development of resistance is very complicated. Resistance mutations have been well characterized for individual agents, but HIV-positive patients are more often on cocktails of drugs rather than one drug. Therefore, the impact of a mutation on multiple drugs must be considered. In addition, if HIV has multiple mutations, the interpretation of more than one mutation with respect to multiple drugs becomes more complex. Finally, mutations can affect multiple drugs, leading to cross-resistance. The computer algorithm that is used to analyze genotypes takes into account primary and secondary mutations, cross-resistance, and the interactions that can occur between mutations to affect the resistance.[140]

Mutations found by genotyping are generally divided into two groups: **primary resistance mutations** and **secondary resistance mutations**. Primary resistance mutations are those that are specific for a particular drug, reduce the susceptibility of the virus for that drug, and appear in the viral genome shortly after treatment with that agent has begun. The mutated enzyme is generally not as active as the normal enzyme, so viral replication is decreased but still occurs. As treatment with the drug continues, secondary or compensatory mutations occur that try to recover the ability of the virus to replicate at a normal rate. The secondary mutations do not affect the susceptibility of the virus to the drug but rather help the virus replicate in the presence of the drug when one of its replication enzymes is not 100% functional.[128] Once a resistance genotype has been identified, the drug therapy of the patient should be changed as soon as possible to avoid the development of secondary mutations in the virus.

Advanced Concepts

For more information about specific mutations and the interactions of mutations, see: http://hiv-web.lanl.gov, http://www.iasusa.org, http://www.hivdb.stanford.edu/hiv/ and references 136 and 137.

The results of genotyping procedures are reported by listing the mutations that have been identified in the protease and reverse transcriptase genes and the impact those mutations will have on each drug in terms of no evidence of resistance, possible resistance, resistance, or insufficient evidence. The mutations are indicated by reporting a change in the amino acid that is coded by the changed codon, where the wild-type amino acid is written followed by the position of the codon that is changed, followed by the new amino acid. For example, a mutation in codon 184 of the reverse transcriptase gene from ATG to GTG results in an amino acid change from methionine to valine, or M184V. This particular mutation makes the virus resistant to lamivudine.[140]

As with all other molecular-based assays, HIV genotyping procedures need to employ adequate quality and contamination controls. The sensitivity of the methods for detecting a minority of virions that contain mutations in the midst of a majority of wild-type virions is an important consideration. The sensitivity of the automated sequencing methods has been reported to be 20%, and the LiPA has a reported sensitivity of 4% (i.e., it can detect as few as 4/100 mutants in the presence of 96/100 wild types).[140] Proficiency testing is available from CAP and Acrometrix. Acrometrix and Boston Biomedica Inc. provide independent control materials for use in genotyping assays.

Hepatitis C Virus

HCV is an enveloped RNA virus that causes viral hepatitis and cirrhosis and is also associated with causing hepatocellular carcinoma. The virus is transmitted parenterally like HIV. Acute infections are often asymptomatic and rarely associated with jaundice; thus, patients with acute HCV infections are usually not diagnosed at this stage. Among HCV-positive patients, 85% will develop chronic hepatitis, and even most of these patients will be asymptomatic. The development of chronic infections is due to the fact that the envelope proteins that are exposed to the host immune response are antigenic and elicit the production of antibodies, but these proteins are encoded by hypervariable regions much like antibody genes themselves, resulting in extensive variation in the envelope proteins and escape of the virus from antibodies.

Diagnosis of HCV infections is similar to the approach used for HIV diagnosis. Serology is used to detect the presence of antibodies against HCV. If the patient has HCV antibodies, the specificity of the antibodies for HCV antigens is measured by Western blot, where the presence of antibodies with multiple HCV-specificities confirms the diagnosis.

Nucleic acid amplification assays for the qualitative detection and quantitation of HCV are performed by a variety of methods, including RT-PCR (Amplicor HCV; Roche Molecular Systems), transcription-mediated amplification (VERSANT HCV RNA qualitative assay; Bayer Diagnostics, Tarrytown, NY), and branched DNA (VERSANT HCV RNA 3.0; Bayer Diagnostics). The qualitative HCV RNA assays are performed on patients with positive HCV antibody results to confirm active infection or on immunocompromised patients (who are often coinfected with HIV) in whom HCV infection is suspected but antibody tests are negative. The quantitative HCV RNA assays are used as for HIV to determine the viral load and to monitor viral replication in response to antiviral therapy. The viral load and the HCV genotype are used to determine the therapeutic protocol, both type of drug(s) as well as duration.[141]

Six genotypes, 1a, 1b, 2, 3, 4, 5, and 6, and 80 subtypes of HCV have been identified. The genotype of HCV present in a given patient determines the treatment protocol that is used on that patient as particular genotypes are associated with certain antiviral resistance patterns.[141] The HCV genotype is determined by analyzing the 5′ untranslated region because it is the most highly conserved region of the genome. Methods available in the laboratory for HCV genotyping are PCR with RFLP analysis and reverse hybridization (Inno-LiPA, Bayer Corp.), cleavase fragment length polymorphism analysis (Third Wave Technologies; Madison, WI), and direct DNA sequencing (TRUGENE; Bayer Corp.). PCR amplicons produced by the Amplicor HCV Test (Roche Diagnostics; Indianapolis, IN) can be analyzed by the Inno-LiPA or TRUGENE methods to determine the genotype.[140] Inno-LiPA and TRUGENE methods are comparable in the identification of HCV genotypes, with

direct comparison studies demonstrating 91%–99% concordance between methods.[140]

Patients who have viral loads greater than 2,000,000 copies/mL of plasma will not respond as well to conventional interferon-ribavirin therapy compared with patients who have fewer than 2,000,000 copies/mL. Determining the genotype of the virus is more critical to predicting treatment outcomes because genotypes 2 and 3 will respond better to treatment than genotype 1.[127] Physicians are looking for a 2 $\log_{10}$ decrease in HCV RNA as an indication of the likelihood that a patient will respond to treatment. Patients who have a 2 $\log_{10}$ decrease in HCV RNA 12 weeks after treatment begins have a 65% chance of responding, defined by the lack of detection of HCV RNA in qualitative assays where the detection limit is 50-100 copies of virus/mL of plasma. In contrast, patients who do not have a 2 $\log_{10}$ decrease in HCV RNA 12 weeks after treatment begins have only a 3% chance of responding.[142]

Summary

The use of molecular methods to detect and/or further characterize some viruses is well entrenched in the workload of the clinical virology laboratory, e.g., HIV and HCV, to the point that clinicians routinely base treatment decisions on the results of these assays. Patient management without these results is negatively affected. For other viruses, molecular-based testing is increasing in significance, in particular for Cytomegalovirus, BK virus, and Epstein-Barr virus detection in transplant or immunocompromised hosts; HBV viral loads; and Human Papillomavirus detection in cervical samples. For other viruses, molecular-based assays are used only in special situations or when traditional diagnostic tests are negative and there is a high index of suspicion that the virus is present, such as for measles, mumps and rubella viruses. Finally, the use of molecular-based tests for some viruses associated with outbreaks has positively impacted the rapid identification of the etiological agent, because the molecular-based methods are faster and more sensitive and specific than traditional laboratory techniques, e.g., for coronaviruses (severe acute respiratory syndrome [SARS]), Norwalk virus, and West Nile virus.

The development of molecular-based assays for the detection, identification, and characterization of viruses will increase over the next few years. The automation is increasing as is the sensitivity and specificity of the assays. In addition, the ability to detect multiple agents in one clinical specimen, as in multiplex PCR, is going to be a great advantage in the diagnosis of respiratory and genital tract diseases in particular. Multiplex PCR assays have been described for the respiratory viruses alone or in conjunction with bacterial respiratory tract pathogens.[53,143,144] Multiplex PCR assays were described earlier in the chapter for the bacterial causes of genitourinary diseases, and multiplex assays have been developed for genitourinary specimens for the detection of bacterial agents as well as viral agents of genitourinary tract disease. While multiplex assays may seem like a "shotgun approach" to microbiology, the ability to differentiate quickly between multiple organisms that can cause the same symptoms from one clinical specimen will enable physicians to treat their patients quickly and effectively, leading to better treatment outcomes and, just as important, reducing the transmission of organisms from inappropriately treated patients.

Fungi

Applications of molecular-based testing for fungal organisms are less numerous than those for bacterial or viral organisms. Fungi are important causes of human disease, especially in immunocompromised patients. Fungal infections are most often diagnosed by direct staining methods and isolation of the causative agent in culture. As for other organisms, traditional smears and cultures are affected by sensitivity, organism viability, and the length of time required for the organism to grow. In addition, laboratory-acquired infections from fungi are a major risk for laboratory personnel. Despite these problems, direct smears and isolation of fungi are still the major method for detecting fungi in clinical samples.

To detect fungi in clinical samples, broad-range PCR and subsequent analysis is most often used. In this assay, primers anneal to DNA sequences that are common to most of the clinically relevant fungi, such as *Candida*, *Aspergillus*, *Rhizopus* and other zygomycetes, and *Histoplasma* and other dimorphic fungi.[145] Once the sequences are amplified, hybridization to species-specific probes or sequencing is used to identify the fungus to the genus or genus and species level.

Fungi growing in culture are typically identified by their microscopic and macroscopic morphologies. For some of the fungi, though, gene probes have been developed to confirm the identity of the organism growing in culture. The AccuProbe assays (GenProbe, Inc., San

Diego, CA) are available for *Histoplasma*, *Blastomyces*, *Coccidioides*, and *Cryptococcus neoformans*. The use of DNA probes for these organisms is faster and less hazardous than determining the microscopic morphology.

Parasites

Parasites are typically detected and identified by morphology directly in clinical specimens. This method of diagnosis is subject to false negatives because of low organism concentrations and depends greatly on appropriately trained personnel. Molecular-based testing has been limited for the parasites mainly because parasites are not a major cause of disease in developed countries. Recognition that travelers from parasite-endemic countries bring the parasite to developed countries and can serve as a reservoir for transmitting the parasite and that expertise in identifying parasites by morphology is declining have made the development of molecular-based assays for parasite detection and identification more of a need than a luxury.

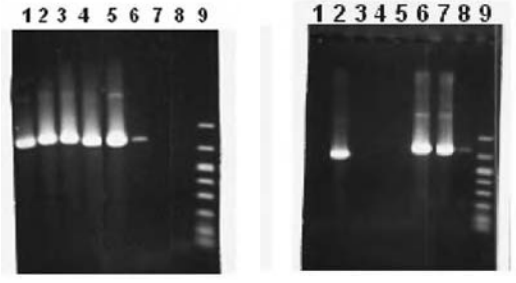
Recently, PCR assays have been developed for the following parasites:[146]

- *Trypanosoma cruzi* in patients with chronic Chagas' disease
- *Trypanosoma brucei* subspecies *gambiense* and *rhodesiense*
- *Plasmodium* in blood and speciation
- *Leishmania* and differentiation to the species level
- *Toxoplasma gondii* in suspected congenital and central nervous system infections
- *Entamoeba histolytica*
- *Cryptosporidium* in water
- Microsporidial detection in stool and small intestine biopsies

The development of multiplex PCR assays to detect multiple parasites in stool samples would be extremely useful. First, multiple parasites can cause diarrhea, and morphology is the only way to differentiate between causative agents. Second, patients can have multiple intestinal parasites at the same time, and laboratory detection of the presence of all parasites is important. Finally, multiple parasites are transmitted in the same water supply; thus, detection of all parasites and appropriate water treatment will reduce large-scale outbreaks of waterborne parasites.

Case Study 12 • 1

During a holiday weekend at a luxury hotel, guests began to complain of stomach-flu with nausea and vomiting. In all, more than 100 of the 200 guests who had dined at the hotel the previous evening described the same symptoms. Eight people had symptoms severe enough to warrant hospitalization. Most, however, recovered within 24–48 hours of the onset of symptoms. Health officials were notified. Interviews and epidemiological analyses pointed to a Norwalk-like virus, or norovirus, infection, probably foodborne. Stool specimens (1–5 ml) and specimens from the suspected food sources (500 mg) were sent for laboratory analysis by RT-PCR. RNA extracted with 1,1,2 trichloro-1,2,2, trifluoroethane was mixed with a guanidium thiocyanate buffer and isolated by organic (phenol-chloroform) procedures. cDNA was synthesized using primers specific to the viral RNA polymerase gene. Strain-specific PCR primers were used to amplify the viral gene. The amplicons, resolved by agarose gel electrophoresis, are shown in the figure below.

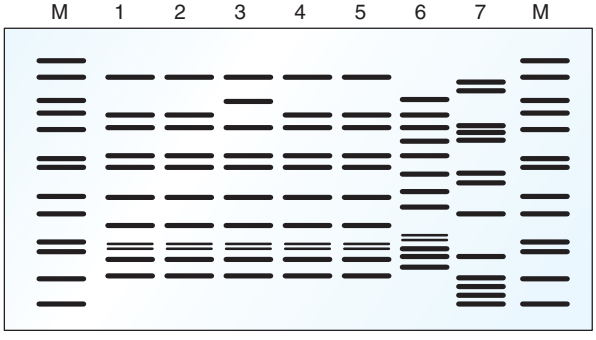


RT-PCR products were resolved on separate gels. Amplicons from four affected individuals (lanes 1–4, left). Lane 5, positive control; lane 6, sensitivity control; lane 7, negative control; lane 8, reagent blank. Specimens from suspected food sources (lanes 1–4, right). Lane 5, salad lettuce from the distributor; lanes 6–8, specimens from three hotel employees working the day of the outbreak; lane 9, molecular weight marker.

1. Do these patients have norovirus?
2. What is the source of the organism?
3. When did the source get contaminated? Did the source come into the hotel infected, or was it infected inside the hotel?

Case Study 12 • 2

Five students from different local community high schools suffered recurrent skin infections with chronic wounds. Nasal swabs and skin specimens from the students were screened for MRSA by inoculation onto Mueller-Hinton agar supplemented with NaCl (0.68 mol/L) containing 6 μg/mL of oxacillin. The cultured organisms exhibited an MIC of more than 32 μg/mL vancomycin and a zone of inhibition of less than 14 mm in diameter. Isolates were sent to the CDC and referred for molecular testing. DNA isolated from the five cases and a control strain were embedded in agarose plugs and digested with *Sma*I for PFGE analysis. A depiction of the gel pattern is shown in the figure below.



PFGE patterns of isolated strains. M, molecular weight markers. Lanes 1–5, isolates from the community infections; Lanes 6 and 7, unrelated hospital isolates.

Further PCR testing was performed on the isolates for virulence factors, particularly for *mecA* gene sequencing and detection of the Panton-Valentine leukocidin (PVL) genes, *lukS-PV* and *lukF-PV*. The results from these tests revealed that all five isolates contained the PVL genes and the type IV *mecA* element.

1. Are all or some of the five isolates the same or different? Which isolates are the same, and which are different? What is the evidence to support your answer?
2. Was there a single source for these organisms or multiple sources?

Case Study 12 • 3

A 39-year-old HIV-positive male has been monitored closely since he was diagnosed as HIV-positive 5 years ago. The man was on HAART and compliant. He was relatively healthy and had not even had a cold in the last 4 years. The man had HIV viral loads as determined by Amplicor RT-PCR, which were always around 10,000 copies/mL, never varying more than 0.2 $\log_{10}$ unit, until the last 6 months, when his viral loads were trending up to 25,500, then 48,900, with his most recent result being 55,000 copies/mL. Genotyping performed on virus isolated from the patient revealed a mutation in the reverse transcriptase gene of M41L that is associated with resistance to zidovudine (AZT).

1. What is the significance of the viral load results over the last 6 months?
2. What is the implication of the genotyping result for the patient's therapy?
3. How should this patient be monitored in the future?

• STUDY QUESTIONS •

1. Which of the following genes would be analyzed to determine whether an isolate of *Staphylococcus aureus* is resistant to oxacillin?
   a. *mecA*
   b. *gyrA*
   c. *inhA*
   d. *vanA*

2. Which of the following methods is a genotypic method used to compare two isolates in an epidemiological investigation?
   a. Biotyping
   b. Serotyping
   c. Ribotyping
   d. Bacteriophage typing

3. For which of the following organisms does caution need to be exercised when evaluating positive PCR

results because the organism can be found as normal flora in some patient populations?
a. *Neisseria gonorrhoeae*
b. HIV
c. *Chlamydophila pneumoniae*
d. *Streptococcus pneumoniae*

4. Which of the following controls are critical for ensuring that amplification is occurring in a patient sample and that the lack of PCR product is not due to the presence of inhibitors?
a. Reagent blank
b. Sensitivity control
c. Negative control
d. Amplification control

5. A PCR assay performed to detect *Bordetella pertussis* on sputum obtained from a 14-year-old girl who has had a chronic cough had two bands, one consistent with the internal control and the other consistent with the size expected for amplification of the *B. pertussis* target. How should these results be interpreted?
a. False positive for *B. pertussis*
b. The girl has clinically significant *B. pertussis* infection
c. *B. pertussis* detection is more likely due to colonization
d. Invalid because two bands were present

6. Which of the following is a disadvantage of molecular-based testing?
a. Results stay positive longer after treatment than do cultures
b. Results are available within hours
c. Only viable cells yield positive results
d. Several milliliters of specimen must be submitted for analysis

7. A molecular-based typing method that has high typing capacity, reproducibility and discriminatory power, moderate ease of performance, and good to moderate ease of interpretation is:
a. Repetitive elements
b. PFGE
c. Plasmid analysis
d. PCR-RFLP

8. A patient has antibodies against HCV and a viral load of 100,000 copies/mL. What is the next test that should be performed on this patient's isolate?
a. Riboyping
b. PCR-RFLP
c. Hybrid capture
d. Inno-LiPA HCV genotyping

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Chapter 13

Lela Buckingham

Molecular Detection of Inherited Diseases

OUTLINE

OBJECTIVES

- Describe mendelian patterns of inheritance as exhibited by family pedigrees.
- Give examples of laboratory methods designed to detect single-gene disorders.
- Discuss nonmendelian inheritance, and give examples of these types of inheritance, such as mitochondrial disorders and trinucleotide repeat expansion diseases.
- Show how genomic imprinting can affect disease phenotype.

Molecular and cytogenetic analyses are a critical component of diagnostic testing, especially for diseases that arise from known genetic events. The identification of a molecular or chromosomal abnormality is a direct observation of the source of some diseases. Methods to detect gene and chromosomal mutations are discussed in Chapters 8 and 9. This chapter will present examples of clinical laboratory tests commonly performed in molecular genetics using these techniques.

The Molecular Basis of Inherited Diseases

Mutations are heritable changes in DNA nucleotide sequences. These changes range from single base pair or point mutations of various types to chromosomal aneuploidy (see Chapter 8). Not all mutations lead to disease.

Polymorphisms are proportionately represented genotypes in a given population (see Chapter 11). Sequence polymorphisms can be located within genes or outside of genes. Benign polymorphisms are useful for mapping disease genes, determining parentage, and identity testing. Balanced polymorphisms can have offsetting phenotypes.

Epigenetic alterations do not change the primary DNA sequence. Epigenetic changes consist of three different forms: DNA methylation, usually alterations of cytosine in CpG islands; genomic imprinting, and chromatin remodeling. DNA methylation shuts down RNA transcription. Genomic imprinting selectively inactivates chromosomal regions, e.g., X chromosome inactivation. Chromatin remodeling sequesters large regions of chromosomal DNA through protein binding and histone modification. Histone modification controls availability of DNA for RNA transcription.

Mutations in germ cells result in inherited disease. Mutations in somatic cells result in cancer and some congenital malformations. Diseases with genetic components are often referred to as **congenital** ("born with") diseases. Congenital disorders are not necessarily heritable, however. Congenital disorders are those present in individuals at birth. Specifically, congenital disorders result when some factor, such as a drug, a chemical, an infection, or an injury, upsets the developmental process. Thus, a baby can have a heritable disease, such as hemophilia, that can be passed on to future generations or a congenital condition, such as spina bifida, that cannot be passed on.

Advanced Concepts

According to the **Lyon hypothesis** (or Lyon's hypothesis) first stated by Mary Lyon in 1961, only one X chromosome remains genetically active in females.[33,34] In humans, one X chromosome is inactivated at random about the 16th day of embryonic development. The inactive X can be seen as a **Barr body** (X chromatin) in the interphase nucleus. Not all X genes are shut off in the inactivated X chromosome. Furthermore, reactivation of genes on the inactivated X occurs in germ cells before the first meiotic division for production of eggs.

Chromosomal Abnormalities

Genome mutations (abnormalities in chromosome number) can be detected by karyotyping, ploidy studies using flow cytometry, and fluorescent in situ hybridization (FISH) studies (see Chapter 8). **Polyploidy** (more than two of any autosome) in animals usually results in infertility and abnormal appearance. Aneuploidy (gain or loss of any autosome) occurs with 0.5% frequency in term pregnancies and 50% in spontaneous abortions. Aneuploidy is usually caused by erroneous separation of chromosomes during egg or sperm production (chromosomal non-disjunction). Autosomal trisomy/monosomy (three copies/one copy of a chromosome instead of two) results from fertilization of gametes containing an extra chromosome or missing a chromosome ($n + 1$ or $n - 1$ gametes, respectively). Autosomal monosomy is generally, but not always, incompatible with life. Sex chromosome aneuploidy is more frequently tolerated, although associated with phenotypic abnormalities.

Mosaicism, two or more genetically distinct populations of cells from one zygote in an individual (in contrast to **chimerism**: two or more genetically distinct cell populations from different zygotes in an individual), results from mutation events affecting somatic or germ cells. Early segregation errors during fertilized egg division occasionally give rise to mosaicism. Mosaicism is relatively common with sex chromosomes, e.g., 45,X/47,XXX (normal female chromosome complement is 46, XX). In this case, later nondisjunction will yield additional popu-

Table 13.1 **Examples of Genome Mutations**

Disorder	Genetic Abnormality	Incidence	Clinical Features
Down's syndrome	Trisomy 21, 47,XY,+21	1/700 live births	Flat facial profile, mental retardation, cardiac problems, risk of acute leukemia, eventual neuropathological disorders, abnormal immune system
Edward's syndrome	Trisomy 18, 47,XY,+18	1/3000 live births	Severe, clenched fist; survival less than 1 year
Patau's syndrome	Trisomy 13, 47,XY,+13	1/5000 live births	Cleft palate, heart damage, mental retardation, survival usually less than 6 mo
Klinefelter's syndrome	47,XXY	1/850 live births	Male hypogonadism, long legs, gynecomastia (male breast enlargement), low testosterone level
XYY syndrome	47,XYY	1/1000 live births	Excessive height, acne, 1%–2% behavioral disorders
Turner's syndrome	45,X and variants	1/2000 live births	Bilateral neck webbing, heart disease, failure to develop secondary sex characteristics, hypothyroidism
Multi X females	47,XXX; 48,XXXX	1/1200 newborn females	Mental retardation increases with increasing X

lations. Rarely, autosomal haploids will be lost with the retention of the triploid lineage, e.g., 45,XY,-21, 46,XY/47,XY,+21 → 46,XY/47,XY,+21. Examples of genome mutations are shown in Table 13.1

Chromosome mutations (abnormalities in chromosome structure) larger than 4 million bp can be seen by karyotyping; smaller irregularities can be seen with the higher resolution of FISH (see Chapter 8). Structural alterations include translocations (reciprocal, nonreciprocal), inversions (paracentric, pericentric), deletions (terminal, interstitial, ring), duplications (isochromosomes), marker chromosomes, and derivative chromosomes.

Structural mutations require breakage and reunion of DNA. Chromosomal breakage is caused by chemicals and radiation. Chromosomal breakage also results from chromosome breakage syndromes, e.g., Fanconi's anemia, Bloom's syndrome, and ataxia telangiectasia. Some aberrations have no immediate phenotypic effect (reciprocal translocations, inversions, some deletions, some insertions). Approximately 7.4% of conceptions have chromosome mutations. Chromosome mutations are observed in 50% of spontaneous abortions and 5% of stillbirths. Examples of diseases arising from inherited chromosome structure abnormalities are shown in Table 13.2

Chromosome translocations are another type of frequently observed structural abnormality. Translocations are usually somatic events (not inherited) and are most commonly seen in cancer (see Chapter 14).

Patterns of Inheritance in Single-Gene Disorders

Most phenotypes result from the interaction of multiple genetic and environmental factors. Some phenotypes, however, are caused by alteration of a single gene. If the phenotype occurs as predicted by mendelian genetics, patterns of inheritance can be established. Patterns of inheritance (**transmission patterns**) are determined by examination of family histories. A **pedigree** is a diagram of the inheritance pattern of a phenotype of family members (Fig 13-1). There are three main transmission patterns: autosomal-dominant, autosomal-recessive, and X-linked or sex-linked recessive. These patterns refer to the disease phenotype.

Table 13.2 **Examples of Chromosomal Mutations**

Disorder	Genetic Abnormality	Incidence	Clinical Features
DiGeorge's syndrome and velocardiofacial syndrome	del(22q)	1/4000 live births	CATCH 22 (cardiac abnormality/abnormal facies, T-cell deficit, cleft palate, hypercalcemia)
Cri du chat syndrome	del(5p)	1/20,000–1/50,000 live births	Growth deficiency, catlike cry in infancy, small head, mental retardation
Contiguous gene syndrome; Wilms' tumor, aniridia, genitourinary anomalies, mental retardation syndrome	del(11p)	1/15,000 live births	Aniridia (absence of iris), hemihypertrophy (one side of the body seems to grow faster than the other), and other congenital anomalies

In **autosomal-dominant** transmission, a child of an affected individual and an unaffected mate has a 50%–100% recurrence risk or likelihood of expressing the disease phenotype (Fig. 13-2). The phenotype of a **loss of function** mutation depends on the type of protein affected. Even though only one copy of a gene is mutated, the mutated protein can interfere with the function of the normal proteins produced from the unmutated chromosome. Complex metabolic pathways are susceptible to loss of function mutations because of extensive interactions between and among proteins. Key structural proteins, especially multimeric complexes, risk dominant negative phenotypes (Fig. 13-3). **Gain of function** mutations are less common than loss of function mutations. In this case, new properties of the mutant allele are responsible for the disease phenotype. Gain of function mutations include gene expression/stability defects that generate gene products at inappropriate sites or times.

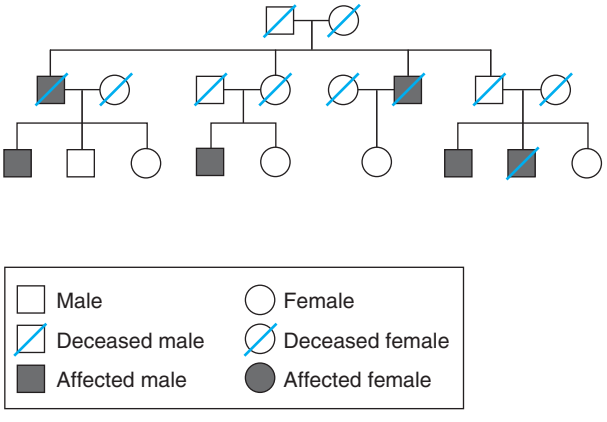


Figure 13-1 A pedigree is a diagram of family phenotype or genotype. The pedigree will display the transmission pattern of a disease.

Autosomal-recessive is the largest category of mendelian disorders. The recurrence risk is 25% if siblings are affected, indicating the presence of the recessive mutation in at least one of the parents. The "margin of safety," that is, having two copies of every gene, requires the loss of the normal allele through somatic events (loss of heterozygosity) or homozygosity for manifestation of the disease phenotype. Autosomal-recessive diseases are more often observed as a result of inbreeding where two individuals heterozygous for the same mutation produce offspring (Fig. 13-4). New mutations are rarely detected in autosomal-recessive transmission patterns. Almost all inborn errors of metabolism are autosomal-recessive.[1] Risk factors for neoplastic diseases, tumor suppressor gene mutations (see Chapter 14), fall in this category.

All **sex-linked** disorders are X-linked because relatively few genes are carried on the Y chromosome. X-linked mutations are almost always recessive. X-linked

Advanced Concepts

Autosomal-dominant mutations can originate from **new mutations** in germ cells; that is, DNA changes that arise in cells that produce eggs or sperm. Establishment of a new mutation as a dominant mutation in a family or in a population is influenced by its effect on reproductive fitness.

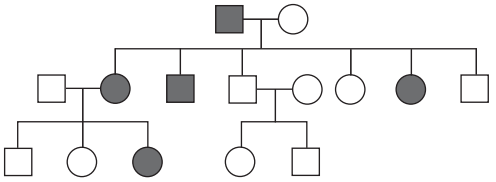


Figure 13-2 In an autosomal-dominant transmission pattern, heterozygous individuals express the affected phenotype (filled symbols).

dominant diseases are rare, e.g., vitamin D–resistant rickets. Even though one X chromosome is inactivated in females, the inactivation is reversible so that a second copy of X-linked genes is available. In contrast, males are **hemizygous** for X-linked genes, having only one copy on the X chromosome. Males, therefore, are more likely to manifest the disease phenotype (Fig. 13-5).

Penetrance is the frequency of expression of disease phenotype in individuals with a gene lesion. **Complete penetrance** is expression of the disease phenotype in every individual with the mutated gene. Complete penetrance is common in recessive phenotypes. **Variable expressivity** is a range of phenotypes in individuals with the same gene lesion. Variable expressivity reflects the interaction of other gene products and the environment on the disease phenotype.

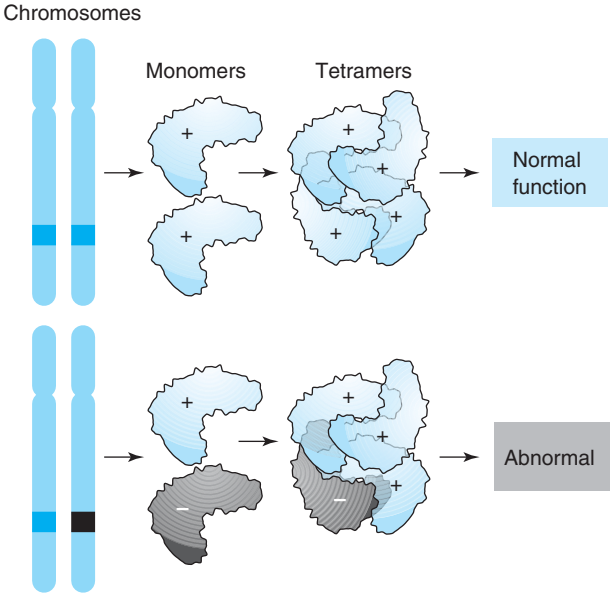


Figure 13-3 Dominant negative mutations affect multimeric proteins. In this illustration, a single mutant monomer affects the function of the assembled tetramer.

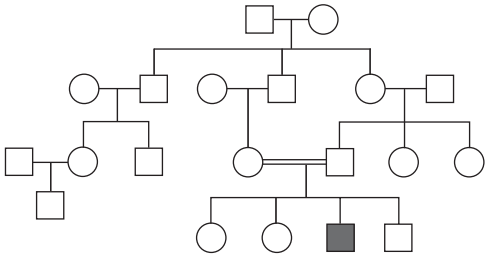


Figure 13-4 Autosomal-recessive mutations are not expressed in heterozygotes. The phenotype is displayed only in a homozygous individual; in this illustration, produced by the inbreeding of two cousins (double horizontal line).

Molecular Basis of Single-Gene Disorders

Single-gene disorders affect structural proteins, cell surface receptor proteins, growth regulators, and enzymes. Examples of diseases resulting from such disorders are shown in Table 13.3. Examples of molecular methods that have been or could be used to detect these gene lesions are also listed. See Chapters 9 and 10 for more detailed explanations of the methodologies. Note that not all of these methods are in common use in molecular diagnostics. Some diseases are effectively analyzed by morphological studies or clinical chemistry. For instance, hemoglobin S is classically detected by protein electrophoresis. Final diagnosis requires physiological, morphological, and laboratory results.

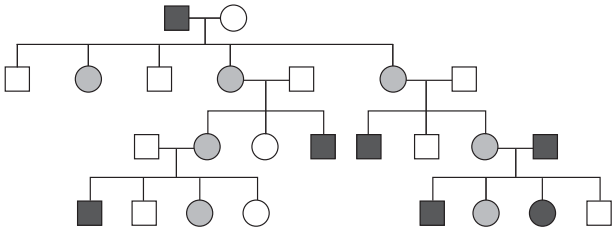


Figure 13-5 X-linked recessive diseases are carried by females but manifested most often in males.

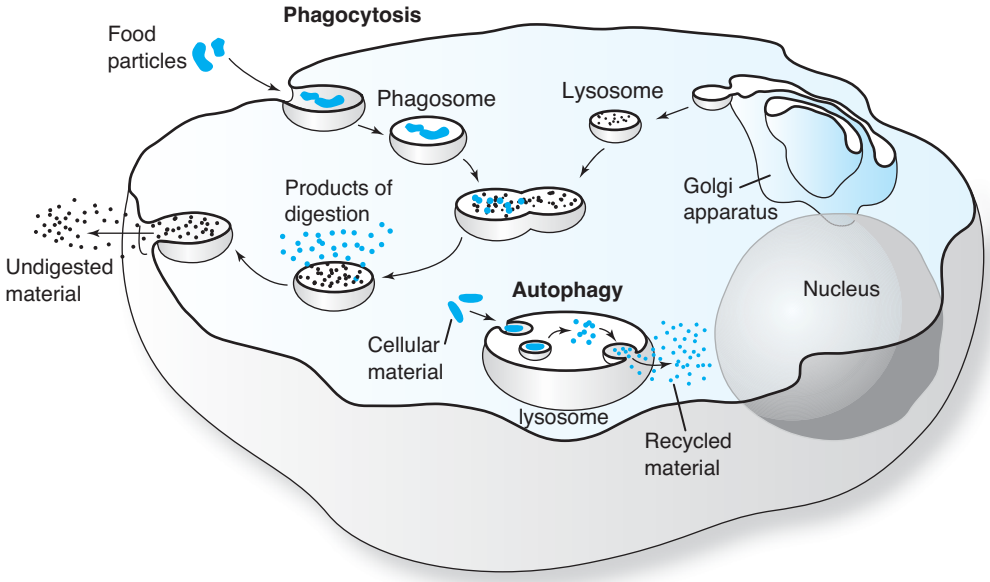
Table 13.3 **Single Gene Disorders and Molecular Methods**

Type of Protein	Type of Disease	Example	Gene (Location)	Type of Mutation	Examples of Molecular Methods
Structural	Hemoglobinopathies	Sickle cell anemia	Hemoglobin beta (11p15.5)	Missense	Sequencing, PCR-RFLP
	Connective tissue disorders	Marfan's syndrome	Fibrillin (15q21.1)	Missense	Sequencing, linkage analysis[20]
	Cell membrane–associated protein dysfunction	Muscular dystrophy	Dystrophin, DMD (Xp12.2)	Deletion	Southern blot RFLP[21], multiplex PCR, linkage analysis
Cell surface receptor proteins	Hypercholesteremia	Familial hypercholesteremia	Low-density lipoprotein receptor (19p13.2)	Deletions, point mutations	Probe amplification,[22] sequencing
	Nutritional disorders	Vitamin D–resistant rickets	Vitamin D receptor (12q12-q14)	Point mutations	Southern blot RFLP, sequencing
Cell growth regulators	Fibromas	Neurofibromatosis type 1 (von Recklinghausen's disease)	Neurofibromin tumor suppressor (17q11.2)	Missense, frameshift, splice site mutations	Sequencing, linkage analysis
	Fibromas	Neurofibromatosis type 2 (von Recklinghausen's disease)	Merlin tumor suppressor, NF-2 (22q12)	Nonsense, frameshift, splice site mutations	Linkage analysis
	Cancer predisposition	Li-Fraumeni syndrome*	p53 tumor suppressor gene, *TP53* (17p13)	Missense mutations	Sequencing, SSCP, DGGE
Enzymes	Metabolic diseases	Alkaptonuria (ochronosis)	Homogentisic acid oxidase (3q21-q23)	Missense, frameshift, splice site mutations	cDNA sequencing,[23] SSCP[24]
		Phenylketonuria	Phenylalanine hydroxylase, PAH or PKU1 (12q24.1)	Splice site, missense mutations, deletions	Ligase chain reaction,[25,26] direct sequencing
	Immunodeficiencies	Severe combined immunodeficiency	Adenosine deaminase (20q13.11)	Point mutations	Direct sequencing, capillary electrophoresis[27]

*A significant proportion of LFS and LFL (Li-Fraumeni–like) kindred do not have demonstrable *TP53* mutations.

Lysosomal Storage Diseases

Lysosomes are cellular organelles in which cellular products of ingestion are degraded by acid hydrolase enzymes (Fig. 13-6). These enzymes work in an acid environment. Substrates come from intracellular turnover (**autophagy**) or outside the cell through phagocytosis or endocytosis (**heterophagy**). Lysosomal storage disorders result from incompletely digested macromolecules due to loss of enzyme function. Storage disorders include defects in any proteins required for normal lysosomal function, giving rise to physical abnormalities. The organs affected depend



■ Figure 13-6 The lysosome is a depository for cell debris. The lysosome contains enzymes that are active in its acid environment to digest proteins delivered from phagocytosis of foreign bodies, endocytosis, and autophagy of internal cellular components such as mitochondria.

on the location and site of degradation of substrate material. Examples of storage diseases are shown in Table 13.4. These disorders are screened by gene product testing; that is, measuring the ability of serum enzymes to digest test substrates. With the discovery of genes that code for the enzymes and their subunits, molecular testing has been used to some extent. Mutations can be detected by direct sequencing, usually after an initial biochemical screening test for loss of enzyme activity.

Table 13.4 **Storage Diseases**

Substrate Accumulated	Disease
Sphingolipids	Tay-Sachs disease
Glycogen	Von Gierke's, McArdle's, and Pompe's disease
Mucopolysaccharides	Hurler, Sheie (MPS I), Hunter (MPS II), Sanfilipo (MPS III), Morquio (MPS IV), Maroteauz-Lamy (MPS VI), Sly (MPS VII)
Mucolipids	Pseudo–Hurler polydystrophy
Sulfatides	Niemann-Pick disease
Glucocerebrosides	Gaucher's disease

Molecular Diagnosis of Single-Gene Disorders

Genetic lesions in single-gene disorders are detected by a variety of hybridization/amplification methods and nucleotide sequencing. Examples of some frequently tested genes are given below.

Factor V Leiden

Discovered in 1994, the Leiden mutation (1691 A→G, R506Q) in the coagulation factor V gene F5 (1q23) causes a hypercoagulable (thrombophilic) phenotype. This genotype is present in heterozygous form in 4%–7% of the general population, and 0.06%–0.25% of the population is homozygous for this mutation. Overall, the annual occurrence of deep venous thrombosis is 1 per every 1000 persons. The disorder is treated with anticoagulants. The risk of thrombosis increases with contraceptive use in women (Table 13.5).

Several approaches have been taken to test for the Leiden mutation. Polymerase chain reaction (PCR) methods include the use of PCR-RFLP (restriction fragment length polymorphism) or SSP-PCR (PCR with sequence-

Table 13.5 Risk of Thrombosis Relative to Normal (1) Under the Indicated Genetic (F5, Prothrombin) and Environmental (OCP) Influences

Status	Risk of Thrombosis
Normal	1
Oral contraceptive (OCP) use	4
Prothrombin mutation, heterozygous	3
Prothrombin mutation + OCP	16
R506Q heterozygous	5–7
R506Q heterozygous + OCP	30–35
R506Q homozygous	80
R506Q homozygous + OCP	100+

specific primers; Figs. 13-7 and 13-8). Nonamplification methods, such as Invader technology, have also been developed to test for this gene mutation.

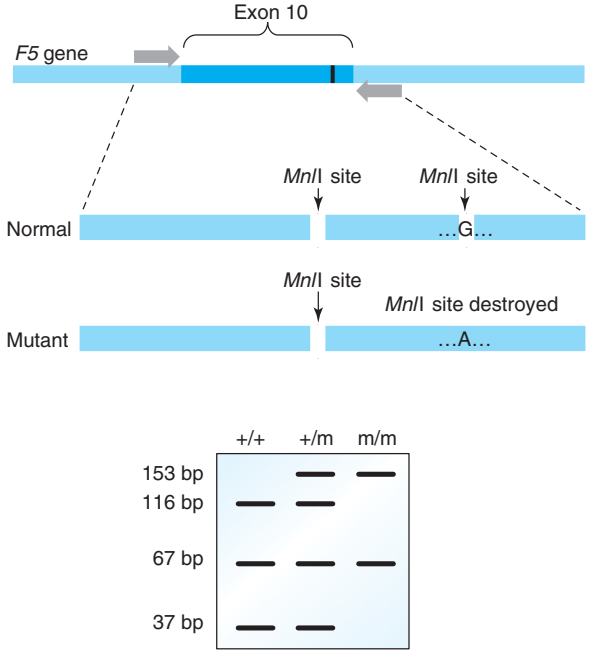


Figure 13-7 PCR-RFLP for the factor V Leiden mutation. The R506Q amino acid substitution is caused by a G to A change in exon 10 of the F5 gene. This DNA mutation destroys an *Mnl* I restriction enzyme site. An amplicon including the site of the mutation, when cut with *Mnl* I, will yield three fragments in normal DNA (+/+) and two products in homozygous mutant DNA (m/m). A heterozygous specimen (+/m) will yield a combination of the normal and mutant pattern.

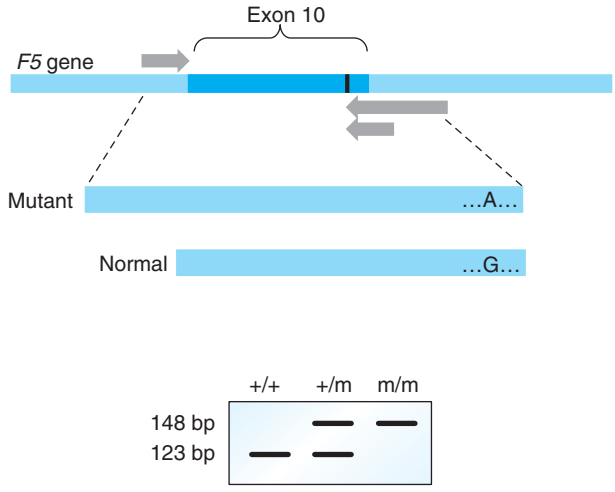


Figure 13-8 In sequence-specific PCR, a primer with thymidine as its final 3′ base will yield a product only if the adenine nucleotide is present. The resulting 148 bp PCR product reflects the presence of the mutation. By designing a primer slightly shorter than but complementary to the normal (G) in the template, a distinct, shorter 123-bp normal product is amplified. In a heterozygous individual, both products will appear.

Prothrombin is the precursor to thrombin in the coagulation cascade and is required for the conversion of fibrinogen to fibrin. A mutation in the 3' untranslated region of the gene that codes for prothrombin or coagulation factor II, F2 (11p11-q12), results in an autosomal-dominant increased risk of thrombosis (see Table 13.5). Laboratories may test for both F2 and F5 mutations. Both may be present in the same individual, in which case the risk of thrombosis is greater than with one of the mutations alone. An example of a multiplex PCR-RFLP method to simultaneously test for both mutations was described previously (see Fig. 9-22). In this method, primers that amplify prothrombin and factor V are designed to destroy or produce *Hind*III restriction sites in the presence of the F5 or F2 mutation, respectively. The sizes of the amplicons and their restriction fragments allow resolution of both simultaneously by agarose gel electrophoresis. The fragment patterns in each lane reveal the F2 and F5 normal or mutant genotypes for each specimen.[2]

Hemachromatosis

Hemachromatosis is an autosomal-recessive condition that causes overabsorption of iron from food. Iron accu-

mulation subsequently causes pancreas, liver, and skin damage, heart disease, and diabetes. Classically, diagnosis is made through measurement of blood iron levels, transferrin saturation, or liver biopsy. This condition is easily treated by phlebotomy.

At the molecular level, hemachromatosis is caused by dysfunction of the hemachromatosis type I gene, HFE or HLA-H (6p21.3). HFE codes for a membrane-bound protein that binds with β_2 microglobulin and transferrin on the membrane of cells in the small intestine and also on the placenta. The protein directs iron absorption based on cellular iron loads. In the absence of HFE function, intestinal cells do not sense iron stores, and iron absorption continues into overload.

The most frequently observed mutation in hemachromatosis is C282Y, present in approximately 10% of the white population, with disease frequency of 2–3 per 1000 people. Other mutations most frequently detected in the HFE protein are H63D and S65C (Fig. 13-9). The C282Y mutation is detectable using PCR-RFLP (Fig. 13-10).

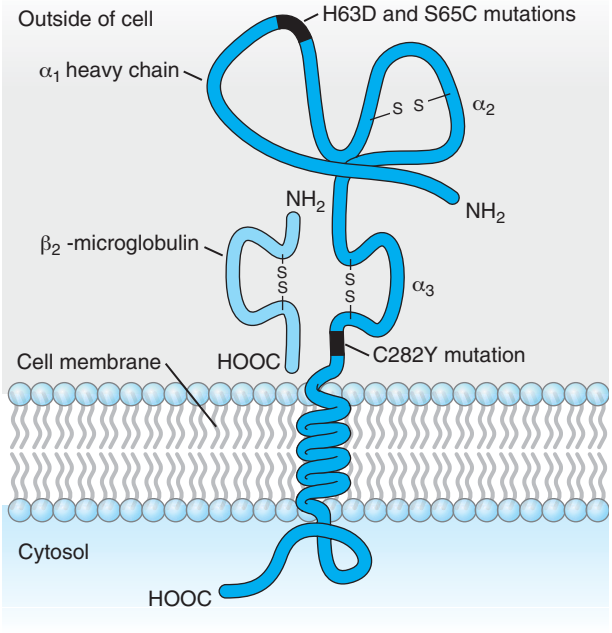


Figure 13-9 The HFE protein is associated with β_2-microglobulin in the cell membrane. The location of the frequently occurring mutations is shown.

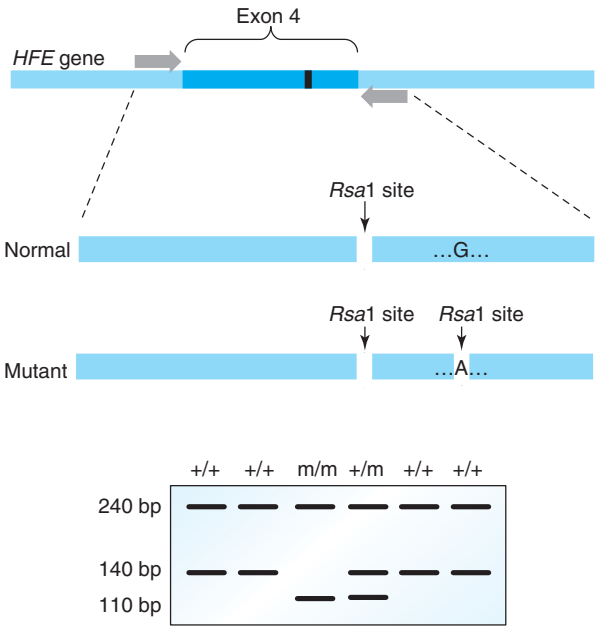


Figure 13-10 Detection of the C282Y mutation by PCR-RFLP. The G→A mutation in exon 4 of the HFE gene produces a site for the restriction enzyme, *Rsa*1. This region is first amplified using primers flanking exon 4 of the gene (arrows). In a normal specimen, the enzyme will produce two fragments, 240 bp and 140 bp. If the mutation is present, the 140-bp normal fragment is cut to a 110-bp and a 30-bp fragment (the 30-bp fragment is not shown). Heterozygous individuals will have both the 140-bp and the 110-bp fragments.

Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal-recessive life-threatening disorder that causes severe lung damage and nutritional deficiencies. With earlier detection by genetic analysis and improved treatment strategies, people with CF can live beyond the fourth decade of life and live more comfortably. CF affects the cells that produce mucus, sweat, saliva, and digestive juices. Normally, these secretions are thin, but in CF a defective gene causes the secretions to become thick and sticky. Respiratory failure is the most dangerous consequence of CF. About 30,000 American adults and children are living with the disorder. Although there is no cure, gene therapy may someday help correct lung problems in people with CF.

CF is caused by loss of function of the CF transmembrane conductance regulator, CFTR gene (7q31.2). The

gene codes for a chloride channel membrane protein. The first and most frequently observed mutation in CFTR is a 3-bp deletion that removes a phenylalanine residue from position 508 of the protein (F508del).[3] More than 1300 other mutations and variations have been reported in and around the CFTR gene in diverse populations. A list of mutations, their locations, and references is available through the Human Genome Variation Society at www.genet.sickkids.on.ca

Genetic testing for CF is important for diagnosis and genetic counseling, as early intervention is most effective in relieving symptoms of the disease. Molecular tests have been designed to detect a variety of mutations that have been described in CF. Methods include RFLP, PCR-RFLP, heteroduplex analysis, temporal temperature gradient gel electrophoresis, single-strand conformation polymorphism (SSCP), SSP-PCR, Invader, bead array technology (Tag-It, TM Bioscience), and direct sequencing. The decisions as to which mutations and how many mutations are tested are still not completely defined. Population differences and variable expressivity influence the choice of mutations to be covered.

Cytochrome P-450

Cytochrome P-450 comprises a group of enzymes localized to the endoplasmic reticulum. These enzymes are mono-oxygenases; that is, they participate in enzymatic hydroxylation reactions and also transfer electrons to oxygen:

$$A\text{-}H + B\text{-}H_2 + O_2 \rightarrow A\text{-}OH + B + H_2O$$

where A is the substrate, B is the hydrogen donor. These enzymes influence steroid, amino acid, and drug metabolism using NADH or NADPH as hydrogen donors. Oxygenation of lipophilic drugs renders them more easily excreted.

The cytochrome P-450 system is present in high concentrations in the liver and small intestine where the enzymes metabolize and detoxify compounds taken in orally. The P-450 system varies from one person to another. This may in part account for different effects of drugs on different people. Hormones, caffeine, chemotherapeutic drugs, antidepressants, and oral contraceptives are examples of compounds affected by these polymorphisms.[4,5] CYP-450 polymorphisms may also compound interactions of multiple drugs taken simultaneously.[6,7]

There are over 30 reported variations of CYP-450 enzymes. Enzymes are classified according to families and subfamilies. For example, CYP2A6 is cytochrome P-450, subfamily IIA, polypeptide 6. CYP1A2 and the enzymes in the CYP2 and CYP3 families are considered to be most important in drug metabolism. Some of the enzymes reported to inhibit or induce drug metabolism include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5-7. The genes coding for these enzymes are located throughout the genome.

Genetic polymorphisms of cytochrome P-450 genes are unequally distributed geographically and in different ethnic populations. Testing for these polymorphisms is used to predict the response to drugs sensitive to metabolism by this enzyme system. In the laboratory, testing for CYP-450 polymorphisms is performed by allele-specific PCR for particular polymorphisms. Multiple P-450 genetic variants may be screened by microarray or bead array technology.

Single-Gene Disorders With Nonclassical Patterns of Inheritance

Mitochondrial mutations, genomic imprinting, and gonadal mosaicism do not follow mendelian rules of inheritance. Mitochondrial mutations are inherited maternally (Fig. 13-11). Genomic imprinting is responsible for specific expression of genes in different cells and tissues. Imprinting is reset at meiosis and fertilization and is different in egg and sperm production.

Gonadal mosaicism is the generation of new mutations in germ line cells. The mutated cells give rise to eggs or sperm carrying the mutation, which then becomes a heritable phenotype. Unusual pedigrees result (Fig. 13-12). Gonadal mosaicism is expected when phenotypically normal parents have more than one affected child, e.g., in osteogenesis imperfecta, an autosomal-dominant phenotype in a child from unaffected parents.

Mutations in Mitochondrial Genes

Mitochondria are cellular organelles responsible for energy production. Mitochondria contain their own ge-

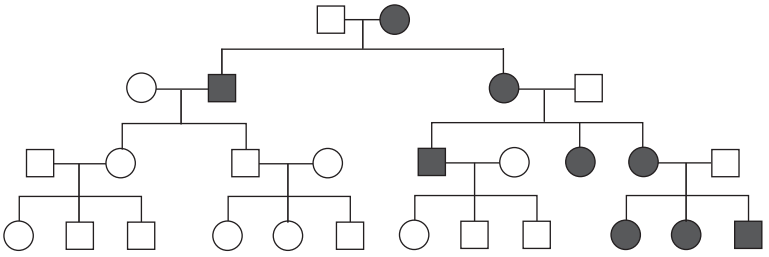


Figure 13-11 Mitochondrial mutations are maternally inherited.

Advanced Concepts

An example of the nature of imprinting is illustrated by comparison of mules and hinnies. A mule is the product of a male horse and a female donkey. A hinny is the product of a female horse and a male donkey. These animals are quite different in phenotype, even though they contain essentially the same genotype. Another illustration of the effects of imprinting is seen in animals cloned by nuclear transfer. Because this process bypasses the generation of eggs and sperm and fertilization, imprinting is not reset, and cloned animals display unexpected phenotypes, such as larger size or early onset of age-related diseases.

nome, a circular DNA molecule 16,569 base pairs in length (Fig. 13-13). The mitochondrial genome contains 37 genes, including a 12S and 16S rRNA, 22 tRNAs, and 13 genes required for oxidative phosphorylation. In addition, the mtDNA contains a 1000-nt control region that encompasses transcription and replication regulatory elements (see Chapter 11). A database of mitochondrial genes and mutations is available at www.MITOMAP.org[8]

Mutations in mitochondrial genes affect energy production and are therefore manifested as diseases in the most energy-demanding organs, muscle and the nervous system.[9,10] Mutations in several genes in the mitochondrial genome have been defined. These mutations result in a number of disease syndromes involving muscular and neurological disorders (Table 13.6).

Mitochondrial mutations are easily detected by a variety of molecular methods. Southern blot is used for detecting large deletions (Fig. 13-14). Point mutations are frequently analyzed by PCR-RFLP (Fig. 13-15). Interpretation of mutation analysis is complicated, however, by the extent of heteroplasmy (mutated mitochondria and normal mitochondria in the same cell) and the nature of the mutation.[11] A range of phenotypes may be present, even in the same family.

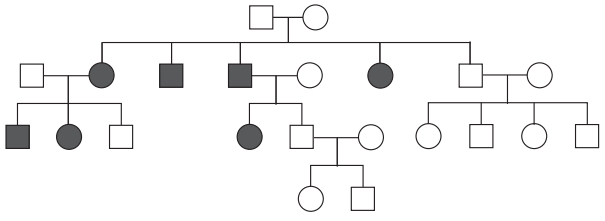


Figure 13-12 Gonadal mosaicism arises as a result of a new mutation. In this example, a dominant disease phenotype has been inherited from two unaffected parents. The mutation is present only in the germ cells of the first-generation parents but is inherited in all cells of the offspring.

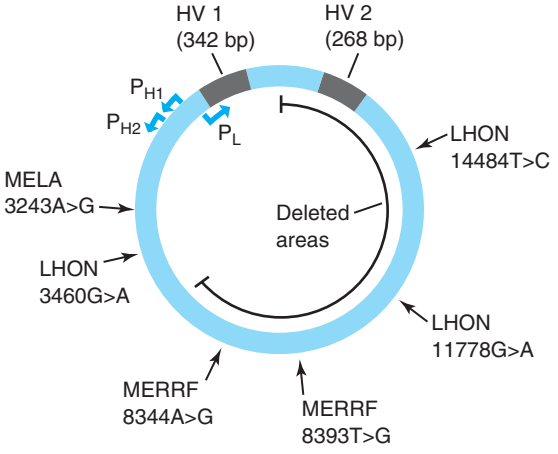


Figure 13-13 Mutations and deletions throughout the mitochondrial genome are associated with muscular and neurological disorders.

Table 13.6 **Diseases Resulting From Mutations in Mitochondrial Genes**

Disorder	Gene Affected	Molecular Methodology
Kearnes-Sayre syndrome	2–7 kb deletions [28]	Southern blot, PCR, PCR-RFLP
Pearson's syndrome	Deletions	Southern blot analysis of leukocytes, PCR-RFLP[29]
Pigmentary retinopathy, chronic progressive external ophthalmoplegia	tRNA (tyr) deletion,[30] deletions	PCR-RFLP, Southern blot analysis of muscle biopsy[31]
Leber's hereditary optic neuropathy	Cyt6 and URF* point mutations	PCR-RFLP
Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes	tRNA (leu) point mutations	PCR-RFLP, sequencing
Myoclonic epilepsy with ragged red fibers	tRNA (lys) point mutations	PCR-RFLP
Deafness		
Neuropathy, ataxia, retinitis pigmentosa (NARP)	ATPase VI point mutation	PCR-RFLP
Subacute necrotizing encephalomyelopathy with neurogenic muscle weakness, ataxia, retinitis pigmentosa (Leigh's with NARP)	ATPase VI, NADH:ubiquinone oxidoreductase subunit mutations	PCR-RFLP
Mitochondrial DNA depletion syndrome	Thymidine kinase gene mutations	PCR, sequencing

*Unknown reading frame

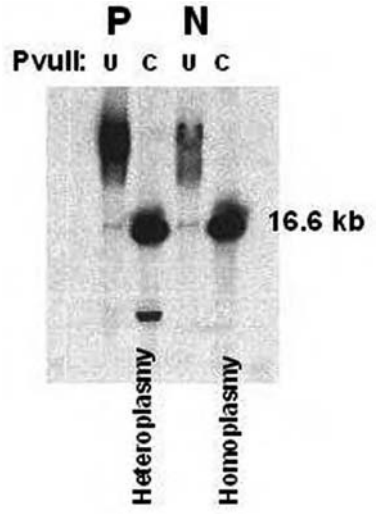


Figure 13-14 A mitochondrial deletion as revealed by Southern blot. DNA was cut with *Pvu*II, a restriction enzyme that cuts once in the mitochondrial genome. The membrane was probed for mitochondrial sequences. Normal mitochondria (N) yield one band at 16.6 kb when cut with *Pvu*II (C). Supercoiled, nicked, and a few linearized mitochondrial DNA circles can be seen in the uncut DNA (U). DNA from a patient with Kearnes-Sayres syndrome (P) yields two mitochondrial populations, one of which has about 5 kb of the mitochondrial genome–deleted sequences. Because both normal and mutant mitochondria are present, this is a state of heteroplasmy. (Photo courtesy of Dr. Elizabeth Berry-Kravis, Rush University Medical Center.)

Genes that control mitochondrial functions are also found on the nuclear genome (Table 13.7). Unlike mitochondrial mutations that display maternal inheritance, these disorders have autosomal patterns of inheritance. Although the causative gene mutation is located on a nuclear gene, analysis of mitochondria may still show deletions or other mutations caused by the loss of the nuclear gene function.

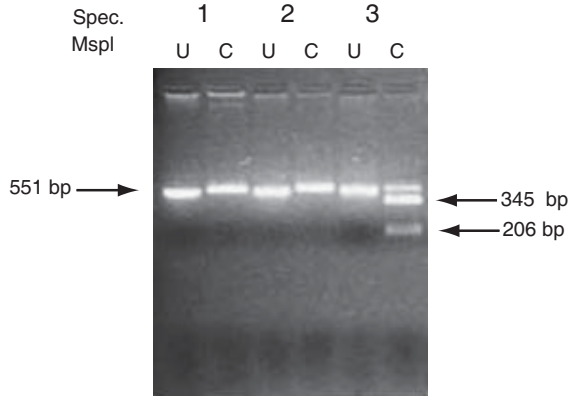


Figure 13-15 Detection of the NARP mitochondrial point mutation (ATPase VI 8993T>C or T>G) by PCR-RFLP. The PCR product was digested with the enzyme *Msp*I (C) or undigested (U). If the mutation is present, the enzyme will cut the PCR product into two pieces, as seen is specimen 3. (Photo courtesy of Dr. Elizabeth Berry-Kravis, Rush University Medical Center.)

Table 13.7 Some Disorders Caused by Nuclear Gene Mutations

Disorder	Gene Affected (Location)	Molecular Methodology
Mitochondrial DNA depletion syndrome	Succinate-CoA ligase, ADP-forming, beta subunit, SUCLA 2 (13q12.2-q13)	Southern blot
Mitochondrial neurogastrointestinal encephalomyopathy	Platelet-derived endothelial cell growth factor, ECGF (22q13-qter)	Sequencing
Progressive external ophthalmoplegia	Chromosome 10 open reading frame 2, C10ORF2 (10q24); polymerase, DNA, gamma, POLG (15q25); solute carrier family 25 (mitochondrial carrier), member 4, SLC25A4/ANT1, (4q25)	Southern blot, SSCP, sequencing[32]

Trinucleotide Repeat Expansion Disorders

Triplet repeats are short tandem repeats (STRs) with 3-bp repeating units (see Chapter 11 for more detailed explanation of STRs). During DNA replication and meiosis, these STRs can expand (or contract) in length. Triplet-repeat mutations may occur in coding and noncoding sequences of genes. The most well-known examples of triplet repeat expansion diseases are fragile X syndrome and Huntington's disease.

Fragile X Syndrome

Fragile X syndrome is associated with a triplet repeat (CGG) expansion in the noncoding region 5' to the fragile X mental retardation gene, *FMR*-1 (Fig. 13-16). The expansion becomes so large in full fragile X syndrome (more than 2000 CGG repeats) that the region is microscopically visible (Fig. 13-17). The CGG repeat expansion 5' to the *FMR*-1 gene also results in methylation of the region and transcriptional shut-down of *FMR*-1.

Symptoms of fragile X syndrome include learning disorders and mental retardation (IQ ~20), long face, large ears, and macro-orchidism (large genitalia). Symptoms are more apparent at puberty. Symptoms increase in severity with each generation in a fragile X family (Fig. 13-18).

In addition to the fragile X chromosome observed by karyotyping, the state of the repeat expansion is also analyzed using PCR and by Southern blot. Although premutations in fragile X carriers are easily detected by PCR, Southern blot is required to detect the full fragile X expansion that could cover thousands of base pairs.

Advanced Concepts

In addition to family history, clinical tests including electroencephalography, neuroimaging, cardiac electrocardiography, echocardiography, magnetic resonance spectroscopy, and exercise testing are important for accurate diagnosis of mitochondrial disorders. High blood or cerebrospinal fluid lactate concentrations as well as high blood glucose levels are observed in patients with some mitochondrial diseases. More direct tests, such as histological examination of muscle biopsies and respiratory chain complex studies on skeletal muscle and skin fibroblasts, are more specific for mitochondrial dysfunction.

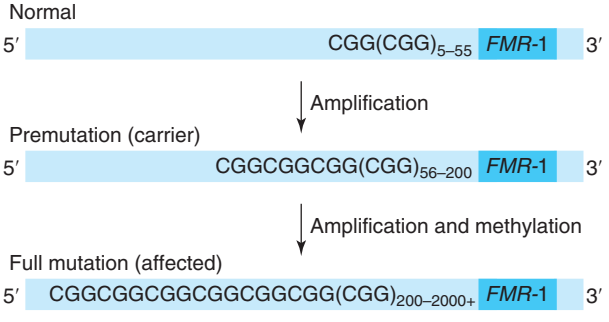
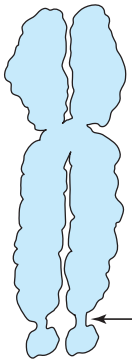
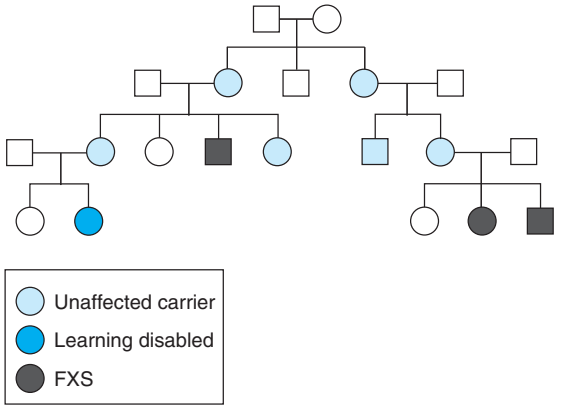


Figure 13-16 Triplet (CGG) repeat expansions in sequences 5′ to the *FMR*-1 gene are observed in fragile X carriers (up to 200 repeats) and fully affected individuals (more than 200 repeats). Normally there are fewer than 60 repeats. Expansion results from amplification of the triplet sequences during meiotic recombination events. The very large repeats (more than 200 repeats) are methylated on the C residues. This methylation turns off *FMR*-1 transcription.



Fragile X chromosome
(in metaphase)

■ Figure 13-17 The fragile X chromosome is characterized by a threadlike process just at the telomere of the long arm (arrow). This is the site of disorganization of chromatin structure by the GC-rich repeat expansions.

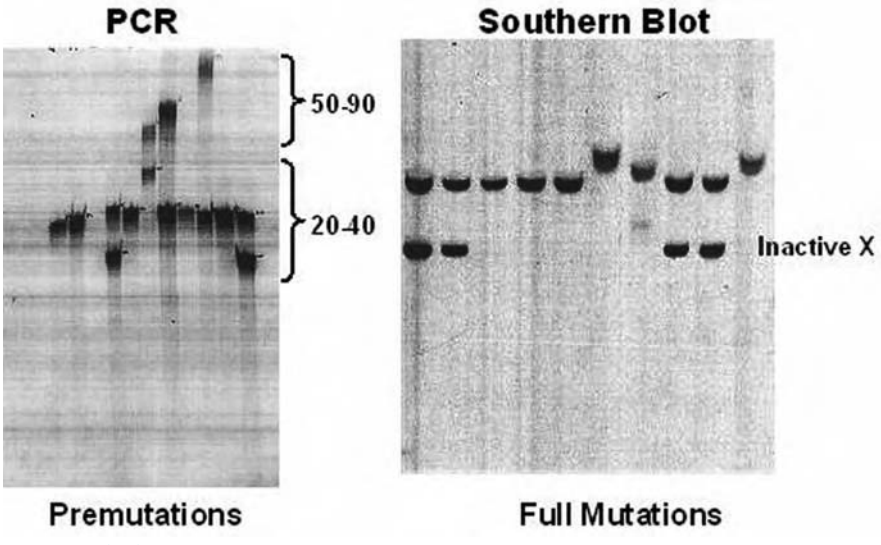


■ Figure 13-18 The symptoms of fragile X syndrome (FXS) become more severe with each generation. The fragile X chromosome cannot be transmitted from fathers to sons but can be transmitted from grandfathers to grandsons through their daughters.

Southern blot also reveals cases of mosaicism where both premutations and full fragile X chromosomes are present in separate cell populations from the same patient (Fig. 13-19).

Huntington's Disease

Huntington's disease, first described by George Huntington in 1872, is associated with expansion within the Huntingtin structural gene (4p16.3). In this repeat expan-



■ Figure 13-19 Detection of premutations by PCR (left) and full fragile X mutations by Southern blot (right). Primers (one of which is labeled with ^{32}P) flanking the repeat region are used to generate labeled PCR products. Premutations appear as large amplicons in the 50–90 repeat range on the autoradiogram at left. Normal samples fall in the 20–40 repeat range. Full fragile X repeats are too large and GC rich to detect by standard PCR. Southern blots reveal full mutations in three of the samples shown. The inactive (methylated) X chromosome in four female patients is detected by cutting the DNA with a methylation-specific restriction enzyme. (Photos courtesy of Dr. Elizabeth Berry-Kravis, Rush University Medical Center.)

Advanced Concepts

The function of the *FMR*-1 gene product is not fully understood. The FMR protein has domains similar to those of RNA binding proteins. Based on these observations, FMR may control expression of other genes by binding to certain RNA structures and preventing translation.[35,36]

sion, the sequence CAG expands from 9–37 repeats to 38–86 in the Huntington gene. The clinical symptoms of Huntington's disease include impaired judgment, slurred speech, difficulty in swallowing, abnormal body movements (chorea), personality changes, depression, mood swings, unsteady gait, and an intoxicated appearance. With an onset in the thirties or forties, these symptoms do not become obvious until the fourth or fifth decade of life, usually after family planning. The child of a person with Huntington's disease has a 50% chance of inheriting the disorder. Genetic counseling, therefore, is important for younger persons with family histories of this disease, especially with regard to having children.

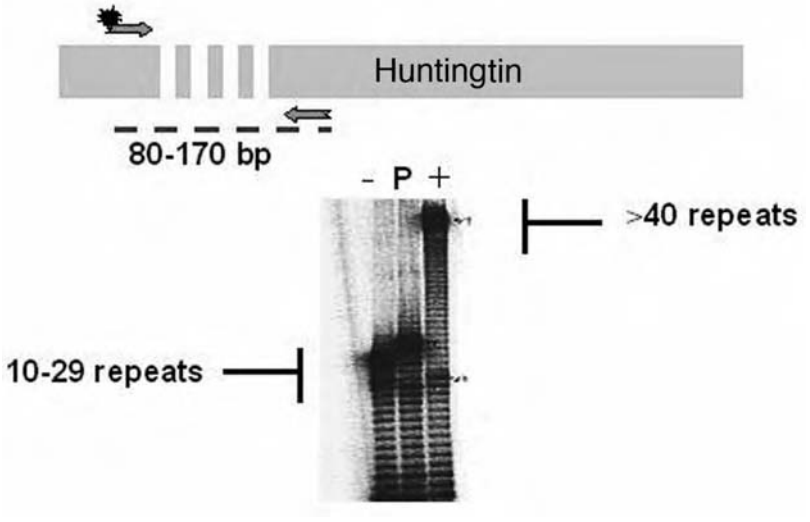
In contrast to fragile X, where the repeat expansion takes place 5' to the coding sequences, the Huntington expansion occurs within the coding region of the gene. The triplet expansion inserts multiple glutamine residues in the 5' end of the Huntingtin protein. This causes the protein to aggregate in plaques, especially in nervous tissue, causing the neurological symptoms seen in this disease.[12] The expansion does not reach the size of the fragile X expansion and is detectable by standard PCR methods (Fig. 13-20).

Idiopathic Congenital Central Hypoventilation Syndrome

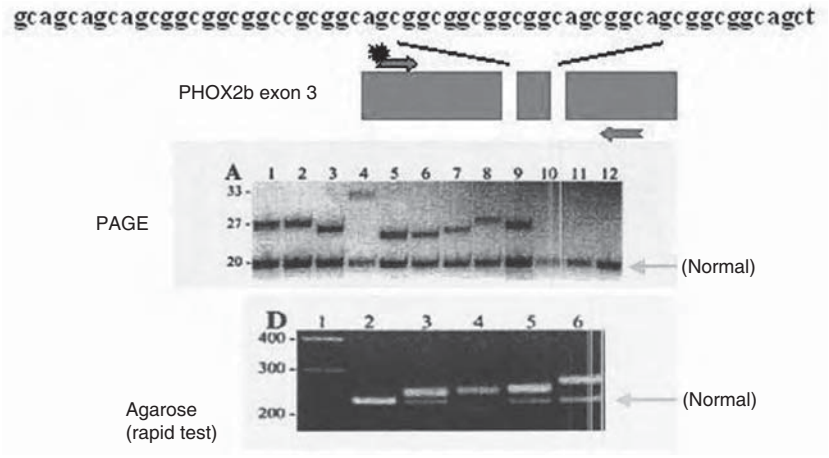
Idiopathic congenital central hypoventilation syndrome (CCHS) is a rare pediatric disorder characterized by inadequate breathing while asleep. More-affected children may also experience hypoventilation while awake. CCHS occurs in association with an intestinal disorder called Hirschsprung's disease and symptoms of diffuse autonomic nervous system dysregulation/dysfunction. A number of gene mutations have been observed in CCHS, including a polyalanine expansion of the paired-like homeobox (*PHOX*2b) gene (4p12)13. The Phox2b protein is a transcription factor containing a domain (homeobox) similar to other proteins that bind DNA.

In CCHS a triplet repeat expansion occurs inside of the *PHOX2b* gene, resulting in the insertion of multiple alanine residues into the protein. The severity of the disease increases with increasing numbers of repeats. The expansion is detected by PCR (Fig. 13-21).[14]

CCHS is usually apparent at birth. In some children, a late onset of the disease occurs at 2–4 years of age. An estimated 62%–98% of patients with CCHS have the *PHOX*2b gene mutation.[15]



■ Figure 13-20 The Huntingtin repeat expansion occurs within the coding region of the Huntingtin gene. The expansion is detected directly by PCR using primers flanking the expanded region (top). A ^{32}P-labeled primer is used, and the bands are detected by autoradiography of the polyacrylamide gel (bottom). In this example, PCR products from the patient (P) fall within the normal range with the negative control (-). The positive control (+) displays the band sizes expected in Huntington's disease. (Photos courtesy of Dr. Elizabeth Berry-Kravis, Rush University Medical Center.)



■ Figure 13-21 The triplet repeat expansion of *PHOX2b* includes triplets that code for alanine (top). The expansion is detected by PCR with a ^{32}P-labeled primer and polyacrylamide gel electrophoresis (center) or by standard PCR and agarose gel electrophoresis (bottom). Normal specimens yield a single PCR product. CCHS specimens yield another larger product in addition to the normal product. The standard PCR test can rapidly show the presence of the expansion, and the PAGE test allows determination of the exact number of alanine codons that are present in the expansion. (Photos courtesy of Dr. Elizabeth Berry-Kravis, Rush University Medical Center.)

Other Triplet Expansion Disorders

Fragile X, Huntington's disease, and CCHS are three of a group of diseases caused by trinucleotide repeat disorders. This category of diseases is subclassified into polyglutamine expansion disorders, which includes Huntington's disease, and nonpolyglutamine expansion disorders, examples of which are listed in Table 13.8.

Genomic Imprinting

Genomic imprinting is transcriptional silencing through histone or DNA modification. Imprinting occurs during egg and sperm production and is different in DNA brought in by the egg or the sperm upon fertilization. The difference is exhibited in genetic disorders in which one or the other allele of a gene is lost.

Uniparental disomy/deletion demonstrates the nature of imprinting on chromosome 15. A deletion in the paternal chromosome 15, del(15)(q11q13), causes Prader-Willi syndrome. Symptoms of this disorder include mental retardation, short stature, obesity, and hypogonadism. Loss of the same region from the maternal chromosome 15 results in Angelman's syndrome, a disorder with very different symptoms, including ataxia, seizures, and inap-

Table 13.8 **Examples of Nonpolyglutamine Expansion Disorders**

Disorder	Gene	Repeat	Expansion, (Normal) – (Symptomatic)*
Fragile XE	Fragile X mental retardation 2 (Xq28)	GCC	(6–35) – (over 200)
Friedreich's ataxia	Frataxin, FRDA or X25 (9q13)	GAA	(7–34) – (over 100)
Myotonic dystrophy	Dystrophia myotonica protein kinase (9q13.2–13.3)	CTG	(5–37) – (over 50)
Spinocerebellar ataxia type 8	Spinocerebellar ataxia type 8 (13q21)	CTG	(16–37) – (110–250)
Spinocerebellar ataxia type 12†	Spinocerebellar ataxia type 12 (5q31–33)	CAG	(7–28) – (66–78)

*The phenotypic effects of intermediate numbers of repeats is not known.
†Although CAG codes for glutamine, this expansion occurs outside of the coding region of this gene and is not translated.

propriate laughter. Both syndromes can occur in four ways: a deletion on the paternal or maternal chromosome 15, a mutation on the paternal or maternal chromosome 15, a translocation with loss of the critical region from one chromosome, and maternal or paternal uniparental disomy in which both chromosomes 15 are inherited from the mother and none from the father or vice versa.

Cytogenetic methods are still used to test for these genetic lesions. Translocations and some deletions are detectable by standard karyotyping. High resolution karyotyping can detect smaller deletions; however, other cases are not detectable microscopically. Newer methods such as FISH with labeled probes to the deleted region[16] can detect over 99% of cases. PCR of RFLP or STR analysis has been used to demonstrate uniparental disomy.[17] As imprinting (DNA methylation) is different on maternal and paternal chromosomes, methylation-specific PCR[18] and Southern blot using methylation-specific restriction enzymes[19] have also been proposed as methods to aid in the diagnosis of these disorders.

Multifactorial Inheritance

Most disorders (and normal conditions) are controlled by multiple genetic and environmental factors. Multifactorial inheritance is displayed as a continuous variation in populations, with normal distribution, rather than as a specific inheritance pattern. Nutritional or chemical exposures alter this distribution. The range may be discontinuous, with a threshold of manifestation. The phenotypic expression is conditioned by the number of controlling genes inherited. The chance of a first-degree relative having a similar phenotype is 2%–7%.

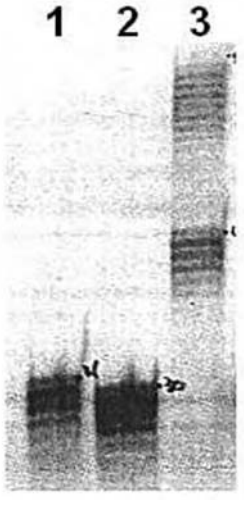
Limitations to Molecular Testing

Although molecular testing for inherited diseases is extremely useful for early diagnosis and genetic counseling, there are circumstances in which genetic testing may not be the optimal methodology. To date, most therapeutic targets are phenotypic so that treatment is better directed to the phenotype. In genes with variable expressivity, finding a gene mutation may not predict the severity of the phenotype. For instance, clotting time and transferrin saturation are better guides for anticoagulant treatment than the demonstration of the causative gene mutations.

Molecular testing may discover genetic lesions in the absence of symptoms. This raises a possible problem as to whether treatment is indicated. Finally, most diseases are caused by dysfunction of multicomponent systems so that several genetic lesions may be present or polymorphic states of other normal genes may influence the disease state. As array technology and haplotype mapping designed to scan at the genomic level are further developed, these methods may contribute to better diagnosis of complex diseases.

Case Study 13 • 1

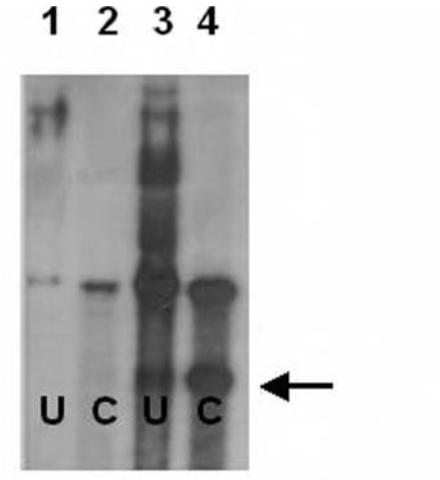
A young mother was worried about her son. Having observed others, she was very aware of how her baby was expected to grow and acquire basic skills. As the child grew, however, he showed signs of slow development. His protruding ears and long face were becoming more noticeable as well. The pediatrician recommended chromosomal analysis for the mother and child. A constriction at the end of the X chromosome was found in the son's karyotype. The mother's karyotype was normal 46,XX. A Southern blot analysis for Fragile X was performed on a blood specimen from the mother, but showed not obvious abnormality. PCR analysis produced the following results:



PCR analysis of the FMR promoter region showing two normal patterns (lanes 1 and 2) and the mother's pattern.

Case Study 13 • 2

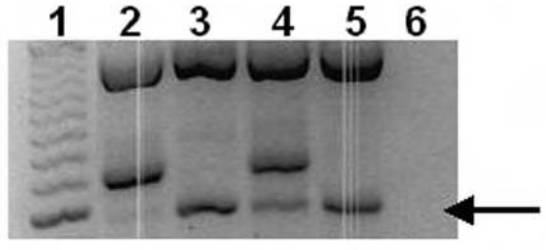
A 14-year-old girl with muscle weakness and vision difficulties (retinopathy) was referred for clinical tests. A muscle biopsy was performed, and aberrant mitochondria were observed in thin sections. Histochemical analysis of the muscle tissue revealed cytochrome oxidase deficiency in the muscle cells. A skeletal muscle biopsy specimen was sent to the molecular genetics laboratory for analysis of mitochondrial DNA. Southern blot analysis of *Pvu*II cut mitochondrial DNA exhibited a band at 16,000 bp in addition to the normal mitochondrial band at 16,500 bp as shown below:



Southern blot of mitochondrial DNA uncut (U) and cut with *Pvu*II (C). Lanes 1 and 2, normal control; lanes 3 and 4, patient. Arrow points to a 16,000 kb band.

Case Study 13 • 3

A 40-year-old man was experiencing increasing joint pain, fatigue, and loss of appetite. He became alarmed when he suffered heart problems and consulted his physician. Routine blood testes revealed high serum iron (900 μg/dL) and 80% transferrin saturation. Total iron binding capacity was low (100 μg/dL). The man denied any extensive alcohol intake. A blood specimen was sent to the molecular genetics laboratory for analysis. The results are shown below:



PCR-RFLP analysis of exon 4 of the *HFE* gene. The PCR product contains 1 recognition site for the restriction enzyme, *Rsa*I. An additional *Rsa*I site is created by the C282Y (G→A) mutation, the most common inherited mutation in hemochromatosis. This extra site results in a fragments of 240 bp, 110 bp (arrow) and ← 30 bp (not shown) instead of the 240 bp and 140 bp normal fragments. Lane 1, molecular weight markers; lane 2, normal control; lane 3, homozygous C282Y; lane 4, heterozygous C282Y; lane 5, patient specimen; lane 6, reagent blank.

Case Study 13 • 4

A 30-year-old woman was brought to the emergency room with a painfully swollen left leg. She informed the nurses that she was taking contraceptives. Deep vein thrombosis was suspected, and compression ultrasound was ordered to look for pulmonary embolism. A clotting test for APC resistance resulted in a 1.5 ratio of clotting time with and without APC. An ELISA test for D-dimer was positive. The patient was immediately treated with heparin, and blood samples were taken. A blood sample was sent to the molecular genetics laboratory to test for the factor V Leiden 1691 G→A mutation and prothrombin 20210 G→A mutations. The results are shown below:

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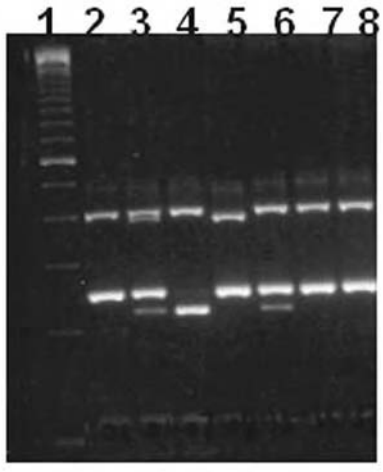

Agarose gel electrophoresis showing the band pattern for prothombin 20210/factor V Leiden mutation detection by multiplex SSP-PCR-RFLP. Specimens were cut with *Hind*III. Lane 1, molecular weight marker; lane 2, normal control (prothrombin, 407 bp + 99 bp, factor V, 241 bp); lane 3, heterozygous control, prothrombin (407, 384, 99, 23 bp)/factor V Leiden (241, 209, 323 bp); lane 4, homozygous factor V Leiden, normal prothrombin; lane 5, homozygous prothrombin 20210 mutation/normal factor V; lane 6, patient specimen; lanes 7 and 8, normal specimens.

• STUDY QUESTIONS •

1. Which of the following is not a triplet repeat expansion disorder?
   a. Fragile X syndrome
   b. Huntington's disease
   c. Factor V Leiden
   d. Congenital central hypoventilation syndrome

2. A gene was mapped to region 3, band 1, subband 1, of the long arm of chromosome 2. How would you express this location from an idiogram?

3. Which of the following can be detected by PCR?
   a. Large mitochondrial deletions
   b. Full fragile X disorder
   c. Mitochondrial point mutations

4. A patient was tested for Huntington's disease. PCR followed by PAGE revealed 25 CAG units.
   a. This patient has Huntington's disease
   b. This patient has a 1/25 chance of contracting Huntington's disease
   c. This patient is normal at the Huntington locus
   d. The test is inconclusive

5. The factor V Leiden mutation can be detected by:
   a. PCR-RFLP
   b. SSP-PCR
   c. Invader technology
   d. All of the above methods

6. The most frequently occurring mutation in the HFE gene results in the replacement of cysteine (C) with tyrosine (Y) at position 282. How is this expressed according to the recommended nomenclature?

7. MELAS is a disease condition that results from an A to G mutation at position 3243 of the mitochondrial genome. This change creates a single *Apa*I restriction site in a PCR product, including the mutation site. What would you expect from a PCR-RFLP analysis for this mutation on a patient with MELAS?
   a. A single PCR product resistant to digestion with *Apa*I
   b. A single PCR product that cuts into two fragments upon digestion with *Apa*I
   c. A single PCR product only if the mutation is present
   d. Two PCR products

8. A father affected with a single gene disorder and an unaffected mother have four children (three boys and

a girl), two of whom (one boy and the girl) are affected. Draw the pedigree diagram for this family.

D16S539, an STR, was analyzed in the family. The result showed that the father had the 6,8 alleles, the mother had the 5,7 alleles. The affected children had the 5,6 and 6,7 alleles, and the unaffected children had the 5,8 and 7,8 alleles.

a. If D16S539 is located on chromosome 16, where is the gene for this disorder likely to be located?
b. To which allele of D16S539 is the gene linked? How might one perform a DNA analysis for the presence of the disorder?
a. Analyze D16S539 for the 6 allele by PCR
b. Sequence the entire region of the chromosome where D16S539 was located
c. Test as many STRs as possible by PCR
d. Use Invader technology to detect the unknown gene mutation.

9. Exon 4 of the *HFE* gene from a patient suspected of having hereditary hemachromatosis was amplified by PCR. The G to A mutation, frequently found in hemachromatosis, creates an *Rsa*1 site in exon 4. When the PCR products are digested with *Rsa*1, which of the following results would you expect to see if the patient has the mutation?
   a. None of the PCR products will be cut by *Rsa*1
   b. There will be no PCR product amplified from the patient DNA
   c. The patient's PCR product will yield extra bands upon *Rsa*1 digestion
   d. The normal control PCR products will yield extra *Rsa*1 bands compared with the patient sample
   *Rsa*1 will not distinguish normal from mutant.

10. Most people with the C282Y or H63D *HFE* gene mutations develop hemachromatosis symptoms. This is a result of:
    a. Iron loss
    b. Excessive drinking
    c. High penetrance
    d. Healthy lifestyle
    e. Glycogen accumulation

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Chapter 14 *Lela Buckingham*

Molecular Oncology

OUTLINE

OBJECTIVES

- Identify checkpoints in the cell division cycle that are critical for regulated cell proliferation.
- List molecular targets that are useful for diagnosisng and monitoring solid tumors.
- Explain how microsatellite instability is detected.
- Describe loss of heterozygosity and its detection.
- Contrast cell-specific and tumor-specific molecular targets.
- Show how clonality is detected using antibody and T-cell receptor gene rearrangements.
- Describe translocations associated with hematological malignancies that can be used for molecular testing.
- Interpret data obtained from the molecular analysis of patients' cells, and determine if a tumor population is present.

Oncology is the study of tumors. A tumor, or **neoplasm**, is a growth of tissue that exceeds and is not coordinated with normal tissue. Tumors are either benign (not recurrent) or malignant (invasive and tending to recur at multiple sites). **Cancer** is a term that includes all malignant tumors. Molecular oncology is the study of cancer at the molecular level, using techniques that allow direct detection of genetic alterations, down to single base pair changes.

Classification of Neoplasms

Cancer is generally divided into two broad groups, solid tumors and hematological malignancies. Solid tumors are designated according to the tissue of origin as **carcinomas** (epithelial) or **sarcomas** (bone, cartilage, muscle, blood vessels, fat). **Teratocarcinomas** consist of multiple cell types. Benign tumors are named by adding the suffix "-oma" directly to the tissue of origin. For example, an adenoma is a benign glandular growth. An adenocarcinoma is malignant.

Metastasis is the movement of dislodged tumor cells from the original (primary) site to other locations. Only malignant tumors are metastatic. No one characteristic of the primary tumor predicts the likelihood of metastasis. Both tumor and normal cell factors are involved. The presence of metastasis increases the difficulty of treatment. Clinical analysis may be performed to detect the presence of relocated or circulating metastasized cells to aid in treatment strategy.

With regard to hematological malignancies, tumors arising from white blood cells are referred to as leukemias and lymphomas. **Leukemia** is a neoplastic disease of blood-forming tissue in which large numbers of white blood cells populate the bone marrow and peripheral blood. **Lymphoma** is a neoplasm of lymphocytes that forms discrete tissue masses. The difference between these diseases is not clear, as lymphocytic leukemias and lymphomas can display bone marrow and blood symptoms similar to those of leukemias. Furthermore, chronic lymphomas can progress to leukemia. Conversely, leukemias can display lymphomatous masses without overpopulation of cells in the bone marrow.

Within lymphomas, **Hodgkin's disease** is a histologically and clinically different disease than all other types of lymphoma, termed **non-Hodgkin's lymphoma** (NHL). **Plasma cell** neoplasms, which arise from terminally differentiated B cells, are also classified in a separate category. Some of the physiological symptoms of plasma cell tumors are related to the secretion of immunoglobulin fragments by these tumors.

Advanced Concepts

As imaging technology advanced, several efforts were made for classification of NHL. The earliest was the Rappaport classification in 1966, developed at the Armed Forces Institute of Pathology. The Keil classification, used in Europe, and the Lukes and Collins classification, used in the United States, were proposed in 1974. In 1982 an international group of hematopathologists proposed the Working Formulation for Clinical Usage for classification of NHL.[139] The Working Formulation was revised in 1994 by the International Lymphoma Study Group, which proposed the World Health Organization/Revised European-American Classification of Lymphoid Neoplasms (REAL). The REAL classification included genetic characteristics in addition to morphological tissue architecture. With increasing ability to detect molecular characteristics of cells, including patterns of gene expression, classification will continue to evolve.

Molecular Basis of Cancer

Cancer is caused by nonlethal mutations in DNA. The mutations affect two types of genes: **oncogenes** and **tumor suppressor** genes. These genes control the cell division cycle (Fig. 14-1).

Oncogenes promote cell division. Oncogenes include cell membrane receptors that are bound by growth factors, hormones, and other extracellular signals. These receptors transduce signals through the cell membrane into the cytoplasm through a series of protein modifications that ultimately reach the nucleus and activate factors that initiate DNA synthesis (move the cell from G1 to S phase of the cell cycle) or mitosis (move from G2 to M). Oncogenes also support cell survival by inhibiting **apoptosis**, or self-directed cell death. More than 100 oncogenes have been identified in the human genome.

Tumor suppressors slow down or stop cell division. Tumor suppressors include factors that control transcrip-

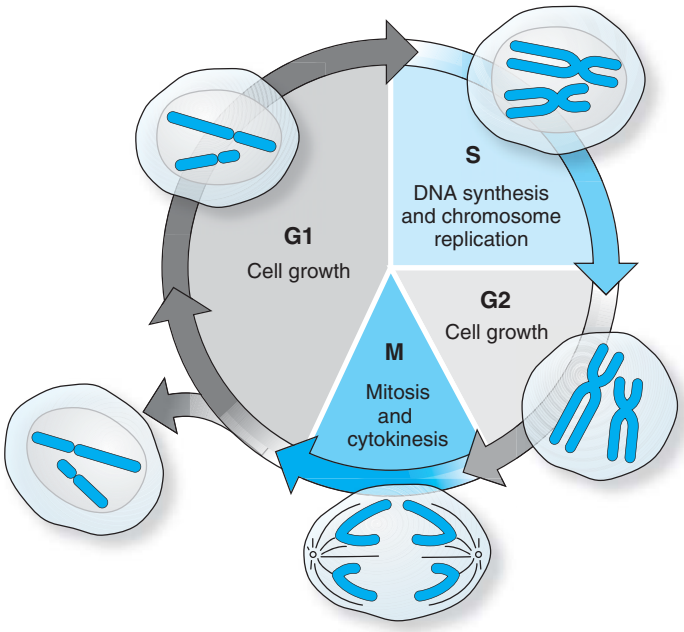


Figure 14-1 The cell division cycle. After mitosis (M), there are two haploid (one diploid) complements of chromosomes (46 chromosomes) in the G1 phase of the cell division cycle. DNA is replicated during the S phase, resulting in four haploid (two diploid) complements in the G2 phase. The chromosomes are distributed to two daughter cells at mitosis each receiving 46 chromosomes. Cancer results when the cell division cycle proceeds from G1 to S or G2 to M phase inappropriately.

tion, or translation of genes required for cell division. Tumor suppressors also participate in repairing DNA damage and in promoting apoptosis. Tumor suppressors counteract the movement of the cell from G1 to S or G2 to M phase. These two points are therefore referred to as the **G1 checkpoint** and **G2 checkpoint** in the cell division cycle. More than 30 tumor suppressor genes have been identified.

In cancer cells, mutations in oncogenes are usually **gain of function** mutations, resulting from amplification or translocation of DNA regions containing the genes or activating mutations that cause aberrant activity of the proteins. Mutations in tumor suppressor genes are usually **loss of function** mutations, resulting in inactivation of the tumor suppressor gene products. These mutations may occur through deletion, translocation, or mutation of the genes. Molecular laboratory testing aids in the diagnosis and treatment strategies for tumors by detecting abnormalities in specific tumor suppressors or oncogenes.

Analytical Targets of Molecular Testing

Although not yet completely standardized, several tests are performed in almost every molecular pathology laboratory. These include tests that target **tissue-specific** or **tumor-specific** genes. Tissue-specific targets are molecular characteristics of the type of tissue from which a tumor arose. The presence of DNA or RNA from these targets in abnormal amounts or locations is used to detect and monitor the presence of the tumor. For example, molecular tests are designed to detect DNA or RNA from cytokeritin genes in gastric cancer, carcinoembryonic antigen in breast cancer, and rearranged immunoglobulin or T-cell receptor genes in lymphoma. Although tissue-specific markers are useful, they are also expressed by normal cells, and their presence does not always prove the presence of cancer.

In contrast, tumor-specific genes are not expressed in normal cells and are, therefore, more definitive with respect to the presence of tumor. Tumor-specific genetic structures result from genome, chromosomal, or gene abnormalities in oncogenes and tumor suppressor genes that are associated with the development of the tumor. Gene mutations and chromosomal translocations are found in solid tumors, leukemias, and lymphomas. Genome mutations, or **aneuploidy**, result in part from the loss of coordinated DNA synthesis and cell division that occurs when tumor suppressors or oncogenes are dysfunctional. Research is ongoing to find more of these

tumor-specific markers to improve molecular oncology testing.

The following sections describe procedures most commonly performed in molecular pathology laboratories; however, due to the rapid advances in this area, the descriptions cannot be all-inclusive. The discussion is divided into solid tumor testing and testing for hematological malignancies. As will be apparent, however, some tests are applicable to both types of malignancies.

Gene and Chromosomal Mutations in Solid Tumors

A number of tests are routinely performed to aid in the diagnosis, characterization, and monitoring of solid tumors. Some of these tests have been part of molecular pathology for many years. Others are relatively new to the clinical laboratory. The methods applied to detect molecular characteristics of tumors are described in Chapters 6–10 of this text.

- Human Epidermal Growth Factor Receptor 2, *HER2/neu/erb-b2 1* (17q21.1)

HER2/neu was discovered in rat neuro/glioblastoma cell lines in 1985.[1] Later it was found to be the same gene as the avian erythroblastic leukemia viral oncogene homolog 2, or *ERBB2,* which codes for a 185-kd cell membrane protein that adds phosphate groups to tyrosines on itself and other proteins (tyrosine kinase activity). This receptor is one of several transmembrane proteins with tyrosine kinase activity (Fig. 14-2). It is very similar to a family of epidermal growth factor receptors that are overexpressed in some cancers[2] (Fig. 14-3).

In normal cells, this protein is required for cells to grow and divide. *HER2/neu* is overexpressed in 25%–30% of human breast cancers, in which overexpression of *HER2/neu* is a predictor of a more aggressive growth and metastasis of the tumor cells. It is also an indication for use of anti-*HER2/neu* antibody drug, **Herceptin** (**trastuzumab**) therapy, which works best on tumors overexpressing *HER2/neu*. Herceptin therapy is indicated presently for women with *HER2/neu*-positive (*HER2/neu* overexpressed) breast cancer that has spread to lymph nodes or other organs.

Of all testing for the overexpression of the *HER2/neu* oncogene, 80%–95% is performed by immunohistochemistry (IHC) using monoclonal and polyclonal antibodies to detect the HER2/neu protein. The HercepTest was developed by Dako and Genentech as a method to define conditions for performance and interpretation of

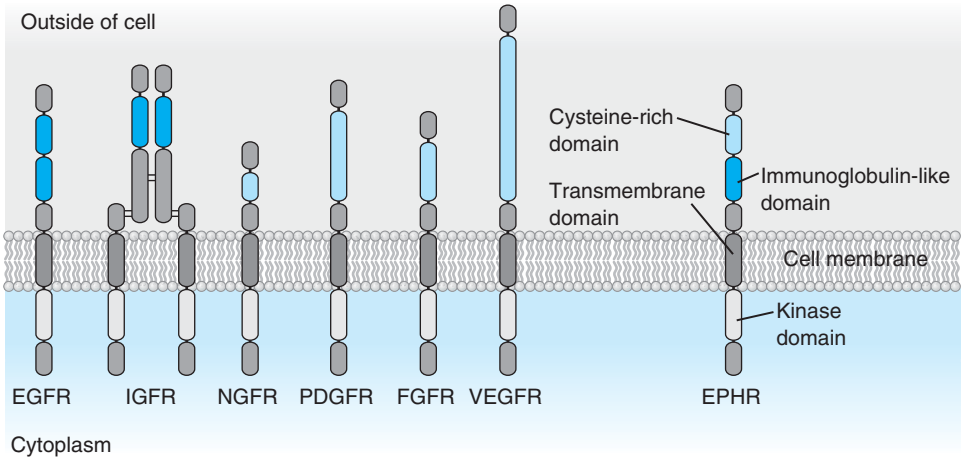


Figure 14-2 Receptor tyrosine kinases include epidermal growth factor receptor (EGFR), insulin growth factor receptor (IGFR), nerve growth factor receptor (NGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), and ephrin receptor (EPHR). These molecules share similarities in that they include a kinase domain, transmembrane domain, cysteine-rich domain, and immunoglobulin-like domain. The EPH receptor has two fibronectin type III domains.

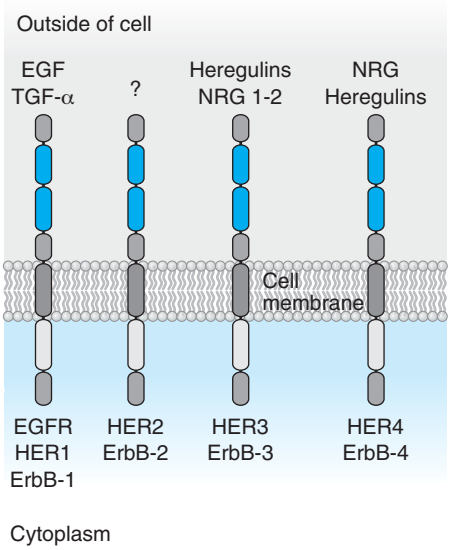


Figure 14-3 The ErbB family of growth factor receptors includes the HER2 receptor and EGFR. The factors that bind to these receptors on the cell surface begin a cascade of events including autophosphorylation and phosphorylation of other proteins by the receptors. These factors include the epidermal growth factor (EGF), human transforming growth factor alpha (TGF-α), heregulins, and neuregulins (NRG). EGF, NRG heregulins are small peptides that are active in the development of various cell types such as gastric mucosa, the heart, and the nervous system.

the IHC test. The IHC test, which is cheaper and more readily available in most institutions, works best on fresh or frozen tissue. Fluorescent in situ hybridization (FISH), which measures DNA and RNA of *HER2/neu*, is more reliable than IHC, especially in older, fixed tissue,[3,4] but is less readily available.

Southern, Northern, and Western studies have shown that *HER2/neu* gene amplification is highly correlated with the presence of increased *HER2/neu* RNA and protein.[5] In contrast to IHC, which measures increased amounts of HER2/Neu protein, FISH directly detects increased copy numbers of the *HER2/neu* gene in DNA. *HER2/neu* gene amplification occurs as a result of tandem duplication of the gene or other genetic events as the tumor cells continue to divide. FISH testing for *HER2/neu* gene amplification requires a labeled probe for the *HER2/neu* gene and a differently labeled control probe for the centromere of chromosome 17. For instance, the PathVysion reagent set (Vysis) includes the HER-2 probe that spans the entire *HER2/neu* gene labeled in Spectrum Orange and a CEP 17 probe that binds to the centromere of chromosome 17 labeled in SpectrumGreen. The copy number of *HER2/neu* relative to the copy number of CEP 17 indicates whether *HER2/neu* is amplified (present in multiple copies on the same chromosome). Data are reported as a ratio of the number of HER2 signals to chromosome 17 centromere signals. A ratio of more than 2 is considered positive or amplified. The number of signals are enumerated in 50–100 cells.

Chromogenic in situ hybridization (CISH) is another method that has been used to detect *HER2/neu* gene amplification[6,7] CISH is a less expensive method that may be more accessible to community laboratories. Using a standard bright field microscope, CISH technology also detects deletions, translocations, or change in the number of chromosomes. An attractive feature of CISH is that the slide images are permanent, facilitating documentation and consultations. Initially, in situ hybridization with chromogenic detection by gene-specific probes was limited by high background and low signal intensity. Newer highly specific probes with repetitive sequences removed, such as Zymed's Subtraction Probe Technology, were designed to address these limitations. CISH has shown 86%–96% concordance with FISH in comparison studies of *Her-2/neu* amplification.[8]

Although FISH and CISH are more accurate and less subjective methods than IHC, IHC is faster, less expensive, and allows the pathologist to assess target gene expression along with other visible landmarks on the slide. Furthermore, protein overexpression (detectable by IHC) can occur without gene amplification (detectable by ISH). Some laboratories use IHC as an initial screening method and then confirm results with FISH or CISH.

- Epidermal Growth Factor Receptor, *EGFR* (7p12)

The epidermal growth factor receptor gene (*EGFR*, *ERBB1*) is another member of the *ERBB* family of growth factor receptors that also includes *ERB3/HER3* and *ERB4/HER4* (see Fig. 14-3). All of these proteins are located in the cell membrane and form dimers with one another upon binding of growth factor from outside the cell (Fig. 14-4). Binding of growth factors evokes the tyrosine kinase activity of the receptors and initiates proliferation signals through the cell cytoplasm.

EGFR is frequently overexpressed in solid tumors. Overexpression has been correlated with poor clinical outcome in research studies. For this reason the EGF

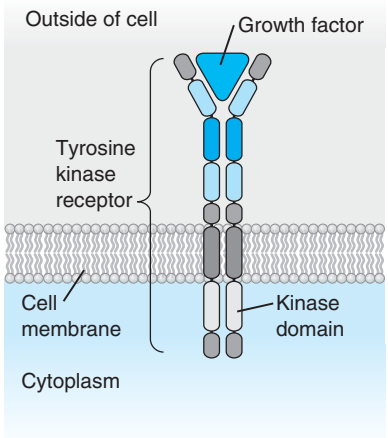


Figure 14-4 Upon binding by epidermal growth factor (EGF), the EGFR receptor in the cell membrane forms a dimer with itself or with other members of the ERBB family of receptors. The dimerization initiates a cascade of events, starting with phosphorylation of the receptor itself catalyzed by the kinase domain.

receptor has been an attractive target for design of therapeutic drugs. Monoclonal antibodies such as cetuximab (Erbitux) have been developed to block ligand (growth factor) binding to the receptor. Agents have also been designed to inhibit the kinase activity of the receptor. Of these tyrosine kinase inhibitors, erlotinib (Tarceva), gefitinib (Iressa), and sunitinib malate (Sutent) have had the most study in clinical trials.

IHC analysis of *EGFR* expression, similar to the testing for *HER2/neu* overexpression, has been used to predict response to monoclonal antibody drugs.[9] The Dako Cytomation EGFR PharmDx test kit has been approved by the United States Food and Drug Administration for this application. Interpretation of the results of *EGFR* expression testing and the predictive value of the test are not always straightforward, however.[10] Quantitative real-time polymerase chain reaction (PCR) and FISH analysis have also been proposed as methods to assess *EGFR* gene copy number.[11]

One possible predictor of response to tyrosine kinase inhibiting agents is specific mutations found in the kinase domain of the protein.[12-15] These mutations can be detected by a number of methods, including sequence-specific PCR, single-strand conformational polymorphism (SSCP), and direct sequencing.[12,16,17] To date, detection of mutations in the EGFR kinase region is not yet approved as a clinical test. Testing for predictors of response or prognosis is complex because several clinical and genetic factors contribute to response to targeted therapies as well as the natural course of the tumor. These include intronic polymorphisms in the *EGFR* gene,[18] expression of other components of the signal transduction pathway,[19,20] or other tumor suppressors such as p53.[21]

- Kirsten rat sarcoma viral oncogene homolog, *K-ras* (12p12), Neuroblastoma ras, *N-ras* (1p13), Harvey rat sarcoma viral oncogene homolog, *H-ras* (11p15)

Signals from extracellular stimuli, such as growth factors or hormones, are transmitted through the cell cytoplasm to the nucleus, resulting in cell proliferation or differentiation (Fig. 14-5). The mitogen-activated protein kinase (MAPK) pathway is a cascade of phosphorylation events that is the major pathway of signal transduction from the cell membrane to the nucleus. Critical compo-

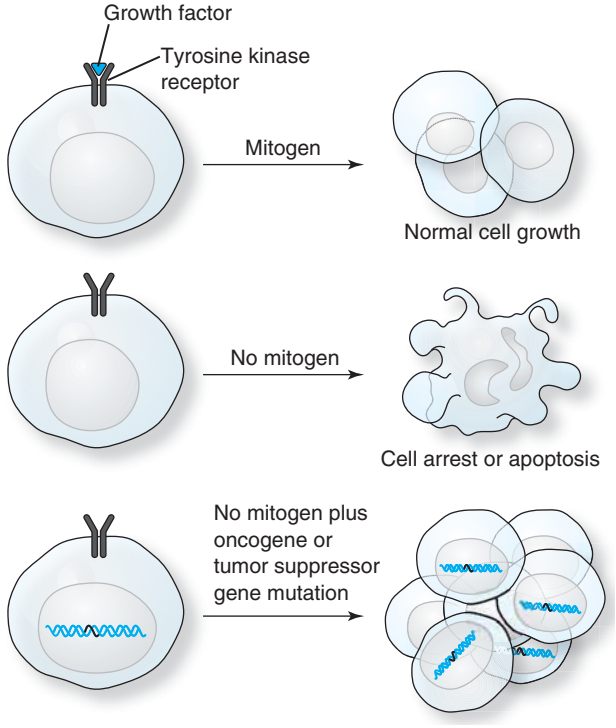


Figure 14-5 Normally, cells grow in the presence of nutrients and factors that stimulate cell division (mitogens). Lack of mitogen stimulation results in cell arrest, or apoptosis. If oncogene or tumor suppressor gene mutations stimulate aberrant growth signals, cells grow in the absence of controlled stimulation.

nents of this pathway are small proteins that bind to GTP in order to become active. These small GTP binding proteins include the *ras* genes that receive signals from the cell surface proteins and activate the initial steps of the MAPK cascade (Fig. 14-6). Mutations in *ras* proto-oncogenes occur in many types of cancers.

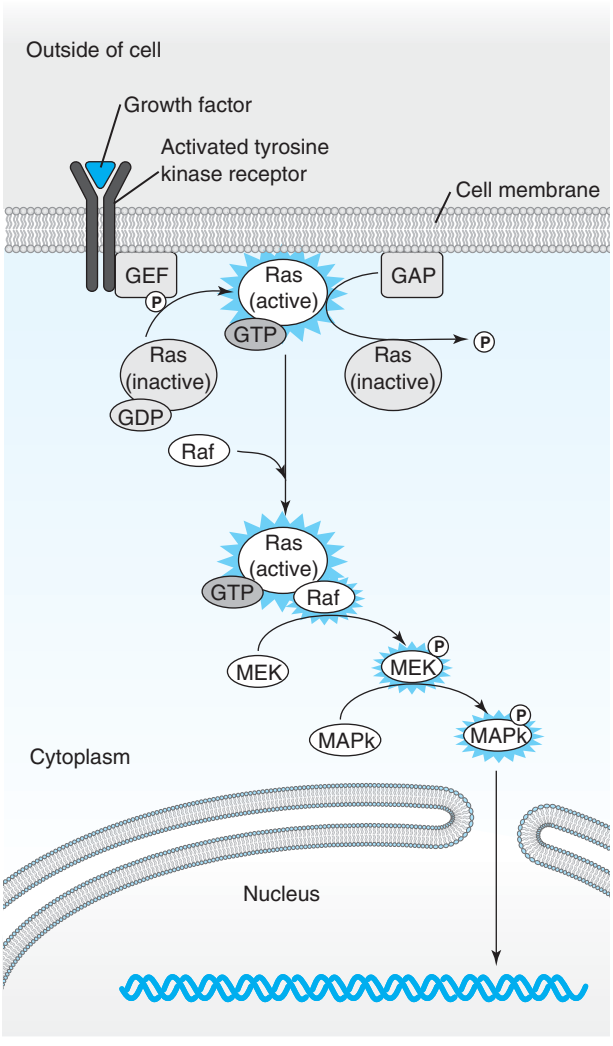


Figure 14-6 Activation of membrane-bound K-ras is initiated by activated receptors bound to mitogens (growth factors or hormones). Active K-ras bound to GTP then initiates a cascade of phosphorylation events that end in the nucleus, where transcription factors modulate gene expression. GDP/GTP exchange on K-ras is modulated by GTPase activating proteins (GAPs), guanosine nucleotide exchange factors (GEFs), and guanosine nucleotide dissociation inhibitors (GDIs).

Advanced Concepts

Regulation of ras GTPase activity is controlled by rasGAP. Several other GAP proteins besides rasGAP are important in signal transduction. Two clinically important proteins of the GAP family of proteins are the gene product of the neurofibromatosis type-1 (*NF1*) locus and the gene product of the breakpoint cluster region (*BCR*) gene. The *NF1* gene is a tumor suppressor gene, and the protein encoded is called neurofibromin. The *BCR* locus is rearranged in the Philadelphia+ chromosome (Ph^+) observed in chronic myelogenous leukemias and acute lymphocytic leukemias.

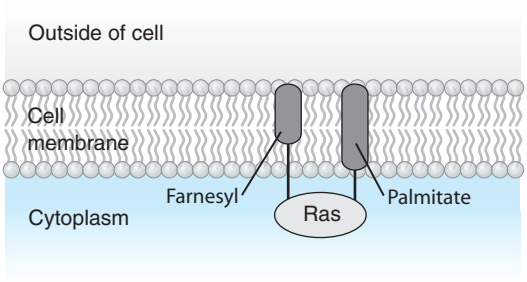
Mammals have three different *ras* genes that produce four similar proteins, K-ras4A, K-ras4B, N-ras, and H-ras. The Ras proteins differ only in their carboxy termini, the end of the proteins that anchor them to the inner surface of the cell membrane (Table 14.1, Fig. 14-7). Because they bind and hydrolyze GTP for energy, the *ras* genes are members of a family of **G-proteins**. The GTP hydrolysis is regulated by **GTPase activating proteins** (GAPs).

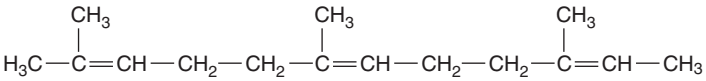
Mutations in *K-ras* are the most common oncogene mutations in human cancers. The most frequently occurring mutations are located in codons 12, 13, 22, and 61 of the *ras* gene. These mutations affect sequences coding for the GTP-binding domain of the protein and throw the Ras

Table 14.1 Four ras Proteins Synthesized From Three Genes, *K-ras, N-ras,* and *H-ras

Protein	Modification	Location
K-ras4A	Farnesylation + palmitoylation	?
K-ras4B	Farnesylation + polybasic aminoacids	Plasma membrane
N-ras	Farnesylation + palmitoylation	Golgi
H-ras	Farnesylation + palmitoylation	Golgi

**K-ras*4A and *K-ras*4B arise from alternate splicing of transcripts of the same gene.





■ Figure 14-7 Ras proteins are anchored to the cell membrane through farnesyl groups (chemical structure at bottom) and palmitoyl groups on the ras proteins. K-ras has only farnesyl groups, whereas N-ras and H-ras have both farnesyl and palmitoyl groups.

protein into a permanently active state that does not require stimulation from GTP hydrolysis. As a result, the Ras proteins harboring these single nucleotide substitutions remain constitutively active in the GTP-bound form.

K-ras mutations are highly correlated with tumor histology and may predict the progress of tumorigenesis in early-stage tumors. Furthermore, the presence of *K-ras* mutations may affect treatment strategy, especially with targeted therapies such as kinase inhibitors and farnesyl transferase inhibitors (K-ras protein is localized to the cell membrane through a farnesyl group). *ras* mutations are detected and indentified by SSCP screening and direct sequencing. Site-specific methods such as pyrosequencing have also been developed. The PyroMark KRAS kit can be used to specifically genotype codons 12 and 13. In addition, mutations in codon 61 can be analyzed using a second primer set provided in the test. A biochip test for detection of multiple specific mutations by hybridization is also available.[22,23]

Advanced Concepts

K-ras must be associated with cellular membranes in order to act. The membrane association is driven by the hydrophobic farnesyl group covalently attached to the ras protein by the enzyme farnesyl transferase. In the absence of farnesyl modification, even mutated ras proteins are not active. Therefore, therapies have been designed to inhibit the farnesylation of ras. These are farnesyl transferase inhibitors.

- Ewing Sarcoma, *EWS* (22q12)

A group of tumors arising from primitive neuroectodermal tissue (PNET)/Ewing's sarcomas comprise a family of childhood neoplasms referred to as the Ewing family. Although immunohistochemical staining for the cell surface enzyho HBA71 (p30/p32MIC2), a neuron-specific enzyme, is helpful in diagnosis of these tumors, no unique characteristics distinguish the different types of tumors that make up this group.

Detection of specific translocations by cytogenetic or molecular methods is useful for diagnostic and prognostic accuracy (Table 14.2). Translocations involving the *EWS* gene at 22q12 (also called *EWSR1* for *EWS* breakpoint region 1) with the *FLI-1* gene at 11q24, t(11;22)(q24;q12) are present in 85% of Ewing's sarcomas. Another translocation between *EWS* and the *ERG* gene at 21q22 is present in 5%–10% of Ewing's sarcomas. Other partners for the *EWS* gene, such as ETV1 at 7p22, E1AF at 17q12, and FEV at 2q33, are present in fewer than 1% of cases.[24,25]

Table 14.2 **EWS Translocation Partners**

Translocation	Tumor
EWS-FLI-1	Ewing's sarcoma, peripheral PNET (72%)
EWS-ERG	Ewing's sarcoma, peripheral PNET (11%)
EWS-WT1*	Desmoplastic small round cell tumor
EWS-ATF1	Clear cell sarcoma

*The *WT1* gene is also associated with Wilms' tumor (WT), one of the common solid tumors of childhood, accounting for 8% of childhood cancers. Several genes or chromosomal areas affect the development of WT: WT1 at 11p13, WT2 at 11p15.5, WT3 at16q, WT5 at 7p15-p11.2, and WT4 at 17q12-q21.

The occurrence of these rearrangements was first revealed by cytogenetics[26,27] and then by PCR methods.[28]

Current laboratory testing at the molecular level involves detection of the tumor-specific translocations by reverse transcriptase polymerase chain reaction (RT-PCR) (Fig. 14-8). Positive results are revealed by the presence of a 120-190–bp PCR product. Negative specimens will not yield a product. As with all assays of this type, an amplification control, such as GAPDH or 18S RNA (see Chapter 7), must accompany all samples to avoid false-negative results. These tests can be performed on fresh, frozen, or paraffin-embedded tissue.[25,29,30] Primers to additional *EWS* translocation partners are used to detect related PNET tumors.

- Synovial sarcoma translocation, chromsome 18—synovial sarcoma breakpoint 1 and 2, *SYT-SSX1, SYT-SSX2* t(X;18)(p11.2;q11.2)

A recurrent reciprocal translocation between chromosome 18 and the X chromosome is found in **synovial sarcoma**, a rare type of cancer of the muscle, fat, fibrous tissue, blood vessels, or other supporting tissue of the body. Synovial sarcoma accounts for 8%–10% of all sarcomas and occurs mostly in young adults. About 80% of cases have the t(X;18) translocation.

The t(X;18) translocation fuses the synovial sarcoma translocation, chromosome 18 gene (*SS18* or *SYT*) with either of two related genes on the X chromosome, synovial sarcoma translocated to X (*SSX1* and *SSX2*). The latter genes are two of five variants, *SSX1, SSX2, SSX3, SSX4,* and *SSX5*. With rare exceptions, only *SSX1* and *SSX2* are fused to *SYT* in the t(X;18) translocation.[31] The fusion gene acts as an aberrant transcription factor, with both activation and repression functions from the *SYT* and *SSX* portions, respectively.

The t(X;18) translocation is detected by FISH or RT-PCR.[32,31] In the latter method, total RNA reverse-transcribed to cDNA is amplified with primers specific for *SSX* and *SYT* genes. In a seminested version of this procedure, the *SSX* primer used in the first round is a consensus primer for both *SYT-SSX1* and *SYT-SSX2*. After the first amplification, *SSX1*- and *SSX2*-specific primers discriminate between the two translocation types. The PCR products are detected by agarose gel electophoresis and ethidium bromide staining. This method can be performed on fresh, frozen, or fixed tissue, depending on the condition of the specimen RNA.

- Paired box-Forkhead in rhabdomyosarcoma, *PAX3-FKHR, PAX7-FKHR*, t(1;13), t(2;13)

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood, accounting for 10% of all solid tumors in children. In addition to alveolar rhabdomyosarcoma (ARMS), there are two additional histo-

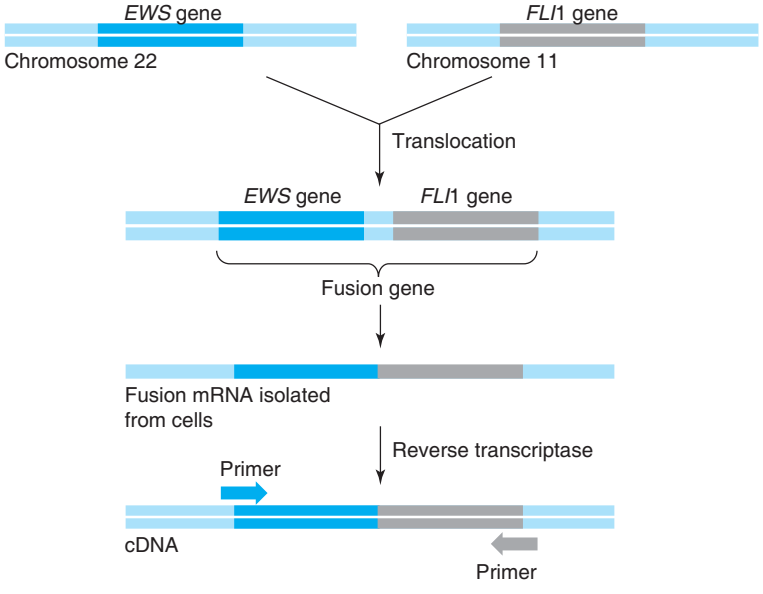


Figure 14-8 RT-PCR is used to detect the t(11:22) mutation in Ewing's sarcoma. One primer is designed to hybridize to the *EWS* gene on chromosome 22 and one primer to the *FLI*1 gene on chromosome 11. If the translocation has occurred, the resulting fusion transcript isolated from the tumor cells will yield cDNA that is amplifiable with the *EWS* and *FLI*1 primers.

logical forms of RMS: embryonal (RMS-E) and primitive (RMS-P). Although histological classification of RMS is sometimes difficult, accurate diagnosis is important for management and treatment of this malignancy because ARMS has a worse prognosis than other subtypes.

Translocations involving the Forkhead in the Rhabdomyosarcoma Gene (*FKHR,* also called *FOXO1A*) and the paired box genes (*PAX3* and *PAX7*) are frequently found in ARMS.[33] The chimeric genes resulting from the translocations encode transcriptional activators with DNA-binding motifs homologous to the forkhead transcription factor first discovered in the fruit fly, *Drosophila*. *PAX-FKHR* translocations have been observed in all subtypes of RMS but are more characteristic of ARMS. Furthermore, *PAX7-FKHR*, t(2;13), is associated with better outcome than *PAX3-FKHR*, t(1;13). Mutations in the *PAX3* gene are also found in Waardenburg's syndrome, a congenital auditory pigmentary syndrome.[34]

The majority of ARMS displays the t(2;13) translocation, with the t(1;13) variant present with 1/3 frequency as t(2;13).[35] Both translocations are detected by FISH, RT-PCR, and real-time PCR.[36-38]

- Tumor protein 53, *TP53* (17p13)

Mutations in *TP53* are found in all types of cancer, and about 50% of all cancers have *TP53* mutations. The gene product of *TP53*, p53, is a 53,000-dalton DNA-binding protein that controls expression of other genes. Normally, p53 participates in the arrest of cell division in the event of DNA damage. The arrest in the G1 phase of the cell cycle allows repair enzymes to correct the DNA damage before DNA synthesis begins. Once the damage is repaired, p53 protein is removed by binding to another protein, MDM2, and degradation. When p53 is not functional, replication proceeds on damaged templates, resulting in the potential for further genetic abnormalities. Also, the mutant protein does not degrade properly and accumulates in the cell nucleus and cytoplasm.

Several studies have shown that mutated *TP53* in tumor tissue is an indicator of poor prognosis in breast, lung, colon, and other types of cancers. The significance of *TP53* status as a predictor of decreased survival time or tumor relapse is, however, controversial.[39] Part of the controversy arises from the different methods used to detect *TP53* mutations. The most common method is detection of the stabilized mutant protein by IHC. Several monoclonal antibodies directed at different epitopes in the p53 protein have been used for this purpose. Because normal p53 protein is transient, significant staining (+2 or above on a scale of 0 to +4) of p53 is considered positive for the mutation.

SSCP (see Chapter 9) and direct sequencing (see Chapter 10) of microdissected tumor tissue are other methods often used to detect *TP53* mutations. Sequencing and SSCP methods cover only exons 5–8 or 4–9 of the *TP53* gene, because these exons encode the regions involved in DNA binding and protein-protein interactive functions of the p53 protein. Screening is routinely performed on frozen or fixed paraffin-embedded tumor tissue.

Both IHC analysis of protein and DNA analysis are used to screen for somatic p53 mutations. A number of studies, however have shown that mutations detected by IHC do not correlate with mutations found by direct DNA analysis.[40-42] There are several explanations for these discrepancies (Table 14.3). Use of microarray technology to screen expression of multiple genes along with *TP53* has been proposed as a more accurate method for predicting survival than either IHC or mutation analysis of *TP53* alone.[43] Methods that include sequencing of the entire *TP53* coding region on cDNA, in combination with IHC, is another accurate approach.[41,44]

In addition to screening for somatic alterations, mutation analysis of *TP53* is also performed to aid in the diagnosis of Li-Fraumeni syndrome, a cancer-prone condition caused by inherited mutations in the p53 gene. In this

Table 14.3 **Sources of Potential Inaccuracies of p53 Mutation Analysis**

Method	False Positive	False Negative
IHC	Staining of normal p53 protein	Deletions or mutations in p53 that remove Ab binding epitopes; promoter mutations
SSCP	Alternate conformers; silent DNA polymorphisms	Less than 5% mutant cells in specimen; mutations outside of the exons screened
Sequencing	PCR mutagenesis; high background	Less than 10% mutant cells in specimen; mutations outside of sequenced area

case, normal tissue will be heterozygous for the mutation, removing the challenge of isolating pure samples of tumor tissue. Once an inherited mutation is detected, further analysis of relatives requires targeting only that mutation.

- Ataxia telangiectasia mutated gene, *ATM* (11q22)

Predisposition to cancer is one symptom of the neurological disease **ataxia telangiectasia** (AT). AT occurs in at least 1/40,000 live births. This disease is caused by mutations in the *ATM* (A-T mutated) gene on chromosome 11. *ATM* mutations are also present in some types of leukemias and lymphomas. Carriers of the autosomal-recessive mutations in *ATM* are at increased risk for developing leukemia, lymphoma, or other types of cancers.

The *ATM* gene product is a member of the phosphatidylinositol-3 kinase family of proteins that respond to DNA damage by phosphorylating other proteins involved in DNA repair and/or control of the cell cycle. The ATM protein participates in pausing the cell cycle at the G1 or G2 phase to allow completion of DNA repair.

Direct DNA sequencing is the method of choice for detection of *ATM* mutations, especially in family members of carriers of previously identified mutations. Another method used is SSCP.[45,46] A functional test for repair of double-strand breaks induced by irradiation is also performed for *ATM*. For this assay, exponentially growing cells are irradiated (1.5 Gy/min), and after 2 hours Colcemid (0.06 μg/mL) is added to inhibit spindle formation. The cells are harvested 2 hours later for Giemsa staining, and the karyotypes are examined. The ratio of aberrations/cell is calculated from the number of chromatid and chromosome breaks (counted as one breakage event) in addition to dicentric chromosomes, translocations, ring chromosomes, and chromatid exchange figures (counted as two breakage events).[47]

If a mutation is identified in a patient with ataxia telangiectasia, other family members may be tested for the presence of the same mutation. Presence of a mutation in family members identifies those with increased risk of AT. Heterozygous carriers of an *ATM* mutation may also be at increased risk for mantle cell lymphoma, B-cell lymphocytic leukemia, or T-cell prolymphocytic leukemia.

- Breast Cancer 1 gene, *BRCA1* (17q21), and Breast Cancer 2 gene, *BRCA2* (13q12)

Approximately 5% of breast cancers result from inherited gene mutations, mostly in the breast cancer genes *BRCA1*[48,49] and *BRCA2*.[50,51] Women who carry a mutation in *BRCA1* have a 60%–80% lifetime risk of breast or ovarian cancer. Men carrying a mutation, especially in *BRCA2*, have a 100-fold increased risk of breast cancer compared with men without a mutation, as well as increased risk of colon and prostate cancer. Both men and women can transmit the mutation to subsequent generations.

The *BRCA1* gene product has a role in embryonic development, and both *BRCA1* and *BRCA2* gene products may also be involved in DNA repair. *BRCA1* and *BRCA2* interact with the RAD51 protein, possibly as a complex to repair damaged DNA.[52]

SSCP, PTT, ddF (see Chapter 9), and other procedures have been used to screen for mutations in these genes. The screening method used for clinical applications, however, is direct sequencing. Three mutations, 187delAG (also called 185delAG) and 5382insC in *BRCA1* and 6174delT in *BRCA2*, occur frequently in particular ethnic populations.[53,54] These known mutations can be easily detected by a number of targeted assays, including sequence-specific PCR and allele-specific oligomer hybridization.

Just as with any genetic analysis, testing for *BRCA1* and *BRCA2* mutations requires thorough patient counseling and education.[55,56] The significance of a *BRCA* mutation will depend on several factors, including penetrance of the gene mutations.[57] If a mutation is not detected in the coding sequences of the genes, the possibility of mutations in the noncoding regions cannot be ruled out.

- Von Hippel-Lindau gene,*VHL* (3p26)

Benign blood vessel tumors in the retina were first reported by Eugen von Hippel, a German ophthalmologist, in 1895. In 1926, Arvid Lindau, a Swedish pathologist, further noted that these retinal tumors were linked to tumors in the blood vessels in other parts of the central nervous system, sometimes accompanied by cysts in the kidneys and other internal organs, and that the condition was heritable. The **Von Hippel-Lindau syndrome** (VHL) is now recognized as a genetic condition involving the abnormal growth of blood vessels in organs, especially those that are particularly rich in blood vessels. It is caused by mutations in the *VHL* gene, which is located on the short arm of chromosome 3. Normally, *VHL* functions as a tumor suppressor gene, promoting cell differ-

entiation. *VHL* may also play a role in sensing hypoxia (low oxygen levels in tissues).[58] VHL syndrome is a predisposition for renal cell carcinoma and other cancers.[59]

Deletions, point mutations, and splice site mutations have been described in patients with VHL. In addition, cases of renal cell carcinoma and tumors of the adrenal gland are accompanied by varied somatic mutations in the *VHL* gene.[60,61] Mutations in the *VHL* gene are detectable by SSCP, and linkage studies have been reported as a method to detect inherited mutations in family members.[62] Direct sequencing, however, is the preferred method of testing for *VHL* gene mutations.

- V-myc avian myelocytomatosis viral-related oncogene, neuroblastoma-derived, *MYCN* or *n-myc* (2p24)

The *n-myc* gene on the short arm of chromosome 2 (2p24) is amplified in cases of neuroblastoma and retinoblastoma. *n-myc* is an oncogene that is counteracted by the tumor suppressor gene, neurofibromatosis type 1 (*NF1*). The *n-myc* gene product is a member of the MYC family of proteins (see *c-myc*).

n-myc gene amplification is detectable by FISH or dot blot analysis.[63-65] Transcription of *n-myc* may also be measured using real-time PCR.[66]

- Rearranged During Transfection (*RET*) proto-oncogene, (10q11)

The *RET* proto-oncogene is located on the long arm of chromosome 10 (10q11). The RET gene product is a membrane tyrosine kinase that participates in sending cell growth and proliferation signals to the nucleus.[67] The *RET* gene is 55 kb in length. The first intron in the gene covers about 24 kb, with exons 2 to 20 contained in the remaining 31 kb. This general structure of a large first intron with small exons is characteristic of tyrosine kinase receptors, such as the KIT, EGFR, and platelet derived growth factor (PDGF) receptors.

Advanced Concepts

The *n-myc* gene encodes a nuclear protein with a basic helix-loop-helix (bHLH) domain that dimerizes with another bHLH protein in order to bind DNA.

The *RET* gene is an example of how different mutations in the same gene result in different diseases. Translocations that result in overexpression of *RET* are found in thyroid papillary carcinomas. Point mutations that activate *RET* (also called *MEN2A*) are found in inherited **multiple endocrine neoplasia** (**MEN**) **syndromes**, a group of diseases resulting in abnormal growth and function of the pituitary, thyroid, parathyroid, and adrenal glands. In contrast, loss of function mutations in the *RET* gene are found in **Hirschsprung's disease**, a rare congenital lack of development of nerve cells in the colon that results in colonic obstruction. Mutations have been reported in about 50% of congenital cases and 20% of sporadic cases of this disorder. Because about 16% of children with congenital central hypoventilation syndrome (CCHS) have Hirschsprung's disease, *RET* mutations were also sought in CCHS. Most of the mutations detected were determined as polymorphic variants, however.[68]

Detection of *RET* gene mutations can aid in diagnosis of MEN diseases.[69,70] Clinical testing targets mainly exons 10, 11, and 16, where most reported mutations have been found. Screening for *RET* gene mutations has been performed by PCR-RFLP, direct sequencing, SSCP, and denaturing gradient gel electrophoresis (DGGE).[70-72]

- Other molecular abnormalities

Increasing numbers of molecular abnormalities are being used to aid in the diagnosis and monitoring of solid tumors. Some examples of potential diagnostic targets are shown in Table 14.4. As molecular aberrations in oncogenes and tumor suppressor genes are identified, molecular analysis becomes more important in their rapid and accurate detection.

Microsatellite Instability

Lynch syndrome, or hereditary nonpolyposis colorectal cancer (**HNPCC**), is an inherited form of colon carcinoma, accounting for about 5% of all colon cancers.[73-76] Predisposition to cancer in this syndrome is caused by mutations in the *MSH2* and *MLH1* genes. Mutations in the *MSH6* and *hPMS2* genes have also been found. These genes are responsible for correcting replicative errors and mismatched bases in DNA, a process called **mismatch repair** (MMR). The MMR system was originally discovered in bacteria (*Escherichia coli*) and further studied in

Table 14.4 Molecular Abnormalities in Some Solid Tumors

Gene	Location	Mutation	Detection Method	Associated Disease
Adenomatous polyposis of the colon	5q21	5q deletion, t(5;10)	Southern blot, FISH, sequencing, SSCP	Familial adenomatous polyposis of the colon
Retinoblastoma (*Rb, RB1*)	13q14.1	13q deletion, t(X;13)	Southern blot, FISH, sequencing	Retinal neoplasm, osteosarcoma
MET proto-oncogene, hepatocyte growth factor receptor	7q31	Missense mutations	Sequencing	Renal carcinoma
KIT proto-oncogene, stem cell factor receptor (*SCFR*)	4q12	Missense mutations	Sequencing	Gastrointestinal stromal tumors
Folliculin (*FLCL, BHD*)	17p11.2	Insertions, deletions in a C8 tract in exon 11	Sequencing	Birt-Hogg-Dube syndrome (hair follicle hamartomas, kidney tumors)
Fumarate hydratase	1q42.1	Frameshift mutations	Sequencing	Hereditary leiomyomatosis, renal cell cancer

yeast (*Saccharomyces cerevisiae*). Similar (homologous) genes were subsequenctly identified in humans and named after the bacterial and yeast genes (Table 14.5). Their gene products form a protein complex that binds to mismatched bases in the DNA double helix or loops formed by replicative errors. At the end of S phase (DNA replication), the system recognizes errors in the newly synthesized daughter strand and uses the template strand, which is methylated, as a guide for repair (Fig. 14-9).

Included in the types of DNA lesions that are repaired by this system are replication errors (RERs) caused by

Table 14.5 Genes of the MMR System

Human Gene	Bacterial Gene	Function
MSH2	*MutS*	Single mismatch, loop repair
MSH3	*MutS*	Loop repair
MSH4	*MutS*	Meiosis
MSH5	*MutS*	Meiosis
MSH6/GTBP	*MutS*	Single mismatch repair
MLH1	*MutL*	Mismatch repair
hPMS2	*MutL*	Mismatch repair (postmeiotic segregation in yeast)
hPMS1	*MutL*	Mismatch repair (postmeiotic segregation in yeast)

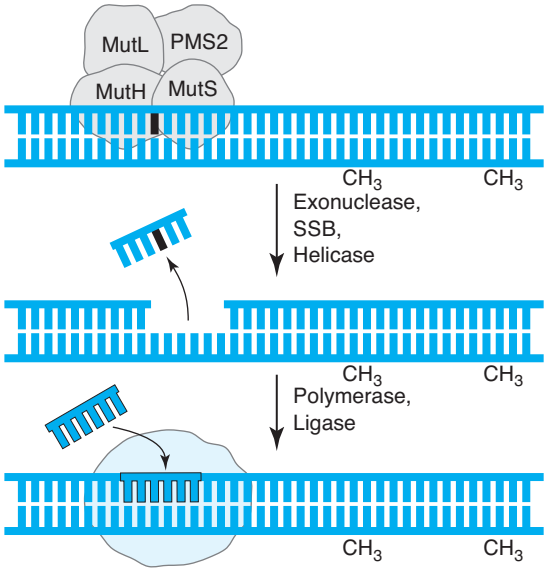


Figure 14-9 The mismatch repair system recognizes a mismatch (top) in the newly synthesized unmethylated strand of DNA. The complex of proteins recruits exonucleases, single-strand DNA binding proteins (SSB), and helicases to remove the erroneous base (center). Polymerases and DNA ligase then replace the missing bases.

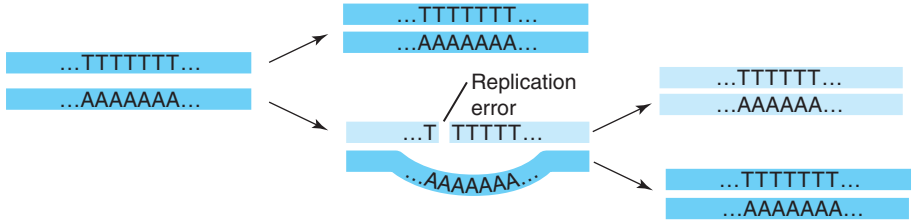


Figure 14-10 Replication errors result from slippage during DNA replication. If the error is not repaired, the next round of replication will create a new allele (top, right) of the original locus. Additional uncorrected errors will produce more alleles.

slippage between the replication apparatus and the DNA template (Fig. 14-10). RERs occur especially in microsatellites where 1–3 nucleotides are repeated in the DNA sequence (see Chapter 11). If the errors remain in the DNA until the next round of replication, new alleles will arise, generating increasing numbers of alleles for the locus, or **microsatellite instability** (MSI). In contrast, a stable locus will retain the same alleles through many rounds of replication. Microsatellite slippage occurs about every 1000 to 10,000 normal cell divisions, most of which are repaired in normal cells. Dysfunction of one or more components of the MMR system will result in MSI, an increase in the number of alleles due to loss of repair. The majority of MMR mutations in HNPCC are found in the *MSH2* and *MLH1* genes. Mutations in *hPMS2* account for fewer than 1%, and mutations in *hPMS1* and *MSH3* are rare. Although direct sequencing of the affected genes is definitive and identifies the specific mutation in a family, the test is expensive and may miss mutations outside of the structural gene sequences or in other genes.[77,78]

About 90% of HNPCC cases display MSI. Because loss of MMR gene function causes MSI, MSI can be used to screen indirectly for mutations in the MMR genes. If a person has inherited a mutation in one copy of an MMR gene, somatic mutation of the remaining copy will result in the MSI phenotype in the tumor cells. MSI, therefore, will be apparent in the tumor where both functional copies of the gene have been lost but not in normal tissue that retains one normal copy of the gene. To perform this test, therefore, normal and tumor tissue from the patient must be compared. MSI is apparent from the increased number of alleles in the tumor tissue compared with that in the normal tissue. MSI is detected by PCR amplification of microsatellite loci and gel electrophoresis (Fig. 14-11) or capillary gel electrophoresis (Fig. 14-12). The detection of instability (more bands or peaks in the tumor tissue compared with the normal tissue) is strong evidence for HNPCC.

The National Cancer Institute has recommended that screening two mononucleotide repeat loci, BAT25 and BAT26, and three dinucleotide repeat loci, D5S346, D2S123, and D17S250, is sufficient for determination of MSI.[79] Alternate markers have been proposed, and some laboratories test additional loci to ensure amplification of at least five loci.[80,81] Further, mononucleotide repeat structures may be more sensitive markers for MSI than dinucleotide repeats, so that some laboratories prefer mononucleotide repeat loci.

If at least two of the five or the majority of loci show instability, the specimen is classified as high instability (MSI-H). Tumors showing MSI in one or a minority of loci tested are classified as low instability (MSI-L). If no MSI is detected in the loci tested, the tumor is stable (MSS). With the present state of clinical correlation, MSI-L and MSS tumors are interpreted as microsatellite-stable, and MSI-H tumors are considered microsatellite-unstable. MSI-H is reported as MSI-unstable with an

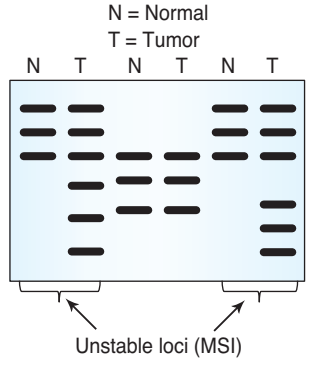


Figure 14-11 Microsatellite instability (MSI) is detected by increased alleles compared with stability at the same locus.

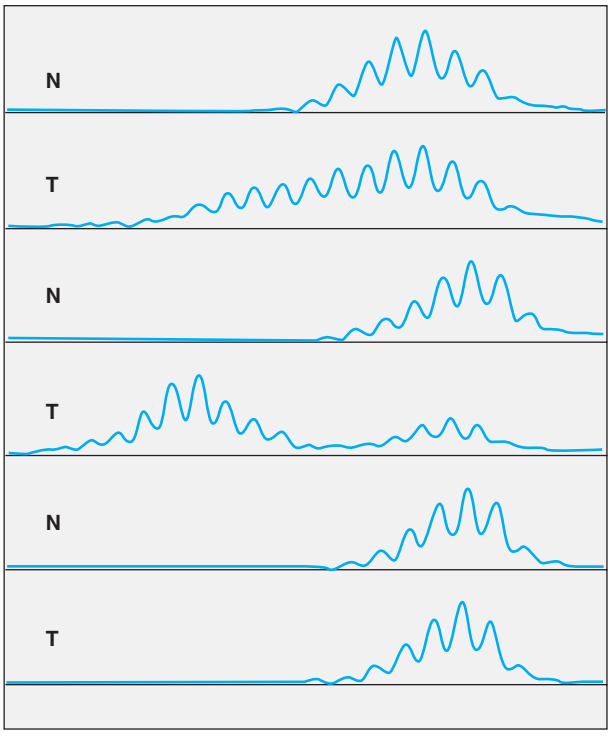


Figure 14-12 MSI detected by capillary gel electrophoresis. DNA from tumor (T) is compared with DNA from normal cells from the same patient (N). Increased alleles in the tumor scans reveal instability at those loci (top four scans). Stable loci look the same in normal and tumor tissue (bottom two scans).

increased likelihood that the patient has HNPCC.[79] If MSI is discovered, the inherited mutation can be confirmed by immunohistochemistry and/or direct sequencing of the *MLH1, MSH2,* and/or *MSH6* genes.[82]

Loss of Heterozygosity

Once a gene mutation is identified in a family, targeted resequencing is used to test for the mutation in other family members. In an inherited condition such as HNPCC or inherited breast and ovarian cancer, blood samples are sufficient for mutation analysis in unaffected individuals. In tumor cells, further testing for **loss of heterozygosity** (LOH) may be performed. LOH reveals the loss of the "good allele" at a locus, uncovering the homologous locus with a recessive mutation. LOH can be detected by PCR amplification of heterozygous STR or variable number tandem repeats (VNTR) loci closely linked to the disease gene. Amplification of loci in tumor cells with LOH will reveal a loss of the allele linked to the normal allele of the gene when compared with the mutant allele (Fig. 14-13). Comparing peak heights in normal (N) and tumor (T) tissues, the formula for LOH is:

$$\frac{\text{(peak height of normal allele in N/peak height of normal allele in T)}}{\text{(peak height of mutant allele in T/peak height of mutant allele in N)}}$$

A ratio of less than 0.5 or more than 2 indicates LOH. Alternately, LOH is assumed if the peak height of the normal allele in the tumor is less than 40% of the height of the normal allele in the normal DNA.

Gene Rearrangements in Leukemia and Lymphoma

Gene rearrangements analyzed for hematological malignancies include **V(D)J recombination**, the normal intrachromosomal rearrangements in B and T lymphocytes as well as the abnormal interchromosomal translocations that can occur in any cell type.

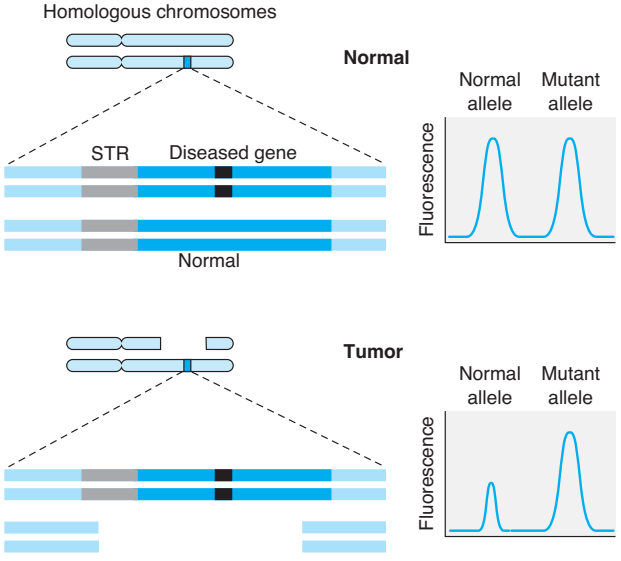
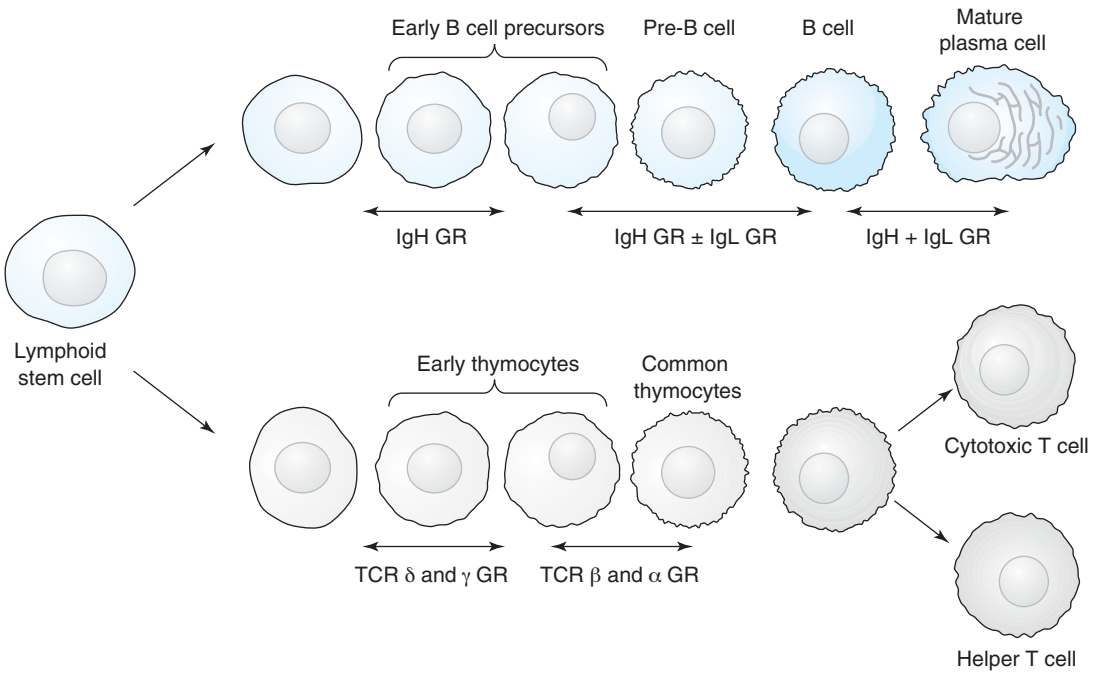


Figure 14-13 Loss of heterozygositiy (LOH) is detected by PCR and capillary electrophoresis of heterozygous STR loci linked to disease genes. A deletion or loss of the normal allele uncovering a recessive mutant allele is identified by the loss of the STR linked to the normal gene (right).



■ Figure 14-14 Gene rearrangements (GR) are normal processes that occur in B and T lymphocytes as they mature from lymphoid stem cells. The genes coding for immunoglobulin heavy and light chains (IgH and IgL, respectively) begin the rearrangement process in early B cells and pre–B cells. The T-cell receptor (TCR) genes rearrange in the order δ, γ, β, and α chains.

V(D)J Recombination

To develop antibody diversity, lymphocytes undergo normal genetic rearrangement of immunoglobulin (Ig) heavy and light chain genes and T-cell receptor genes (Fig. 14-14). The **gene rearrangement** process is a series of intrachromosomal recombination events mediated by recombinase enzymes that recognize specific sequences flanking the gene segments. This process occurs independently in each lymphocyte, so that a repertoire of antibodies is available to match any random invading antigen.

Immunoglobulin Heavy Chain Gene Rearrangement in B Cells

Each antibody consists of two heavy chains and two light chains. The locus coding for the immunoglobulin heavy chain is located on chromosome 14. The unrearranged, or **germline**, configuration of the immunoglobulin heavy chain locus consists of a series of gene segments or repeated exons coding for the functional parts of the antibody protein (Fig. 14-15). These include 123–129 variable (V_H) regions (38–46 functional gene segments) and 9 joining (J_H) regions (6 functional), one of which will connect one variable region with a constant (C_H) region of the antibody or receptor. There are 11 constant regions (9 functional). The immunoglobulin heavy chain gene also contains 27 diversity (D_H) regions (23 functional), one of which will connect the variable and joining regions. The V segments are each preceded by a leader region (L). The leader region codes for a short sequence of amino acids found on the amino terminus of the protein that mark the antibody for secretion or membrane insertion.

As B lymphocytes mature, selected gene segments are joined together so that the rearranged gene contains only one of each V_H, D_H, and J_H segment (see Fig. 14-15). Initially, one D_H and one J_H segment are joined together. The DNA between the two segments is looped out and lost. The D_H-J_H rearrangement occurs in both alleles of the heavy chain gene locus on both chromosomes. Then in only one allele, a V_H segment is chosen and joined to

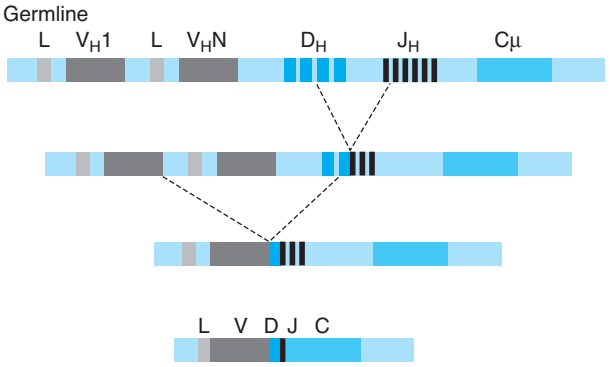


Figure 14-15 Immunoglobulin heavy chain gene on chromosome 14 consists of a series of variable (V), diversity (D), and joining (J) gene segments (germline configuration). The V segments are accompanied by a short leader region (L). One of each type of segment, V, D, and J, is selected and combined by an intrachromosomal recombination event, first D and J, and then V and D. The C (constant) segments are joined through splicing or a secondary recombination event, class switching.

the D_H segment. The completion of the gene rearrangement process on only one of the two immunoglobulin heavy chain gene alleles is referred to as **allelic exclusion**. The rearrangement on the other chromosome will proceed if the first rearrangement fails or is not productive.

When the DNA is cut in the process of the gene rearrangement, terminal deoxynucleotidyl transferase may add nucleotides at the V-D-J junctions, further diversifying the coding sequences of individual antibody genes. After the V(D)J rearrangement occurs, the gene is transcribed, and one of the constant regions is joined to the final messenger RNA by splicing or, alternatively, by a secondary recombination event (**class switching**). The maintenance of the constant regions in the DNA allows for antibody-type switching during the immune response.

Advanced Concepts

The recombination events of the gene rearrangement are mediated by **recombination activating genes** *RAG*1 and *RAG*2. These genes code for enzymes that recognize short **recombination signal sequences** in the DNA where they form a complex that initiates the cutting and religating of the DNA.

Advanced Concepts

The constant region determines the **isotype** of the antibody IgM, IgD, IgG, IgE, or IgA. Each cell can make only one heavy chain protein, although the isotype of the heavy chain may change. A mature B cell will initially produce IgD and some membrane IgM that will migrate to the cell surface to act as the antigen receptor. Upon antigen stimulation, the B cell will differentiate into a plasma cell expressing large amounts of secreted IgM. Some cells will undergo a **class-switch recombination**, placing the VDJ gene next to the genes encoding the IgG, IgE, or IgA constant regions. The B cells will express a different isotype during the secondary response. Most commonly, IgM (primary response) gives way to IgG (secondary response). Production of IgE or IgA instead of IgG can also occur, although the regulation of these switching events is not well understood. Class switching is mediated by different recombinase enzymes than those responsible for VDJ recombination.

Immunoglobulin Light Chain Gene Rearrangement in B Cells

Like the Ig heavy chain gene on chromosome 14, the Ig light chain genes consist of a series of gene segments in the germline configuration (Fig. 14-16). Two separate genes code for the Ig light chains, the kappa locus on chromosome 2 and the lambda locus on chromosome 22. At the kappa locus, there is a single constant gene segment, 5 joining (J_κ) gene segments and at least 76 variable (V_κ) gene segments (30–35 functional) belonging to 7 sequence-related families.[83,84] In addition there is a **kappa deleting element** (KDE) located 24 kbp 3' to the constant region. This element determines deletion of the Igκ constant region in cells producing Ig lambda light chains. The Ig lambda gene locus consists of 52 variable

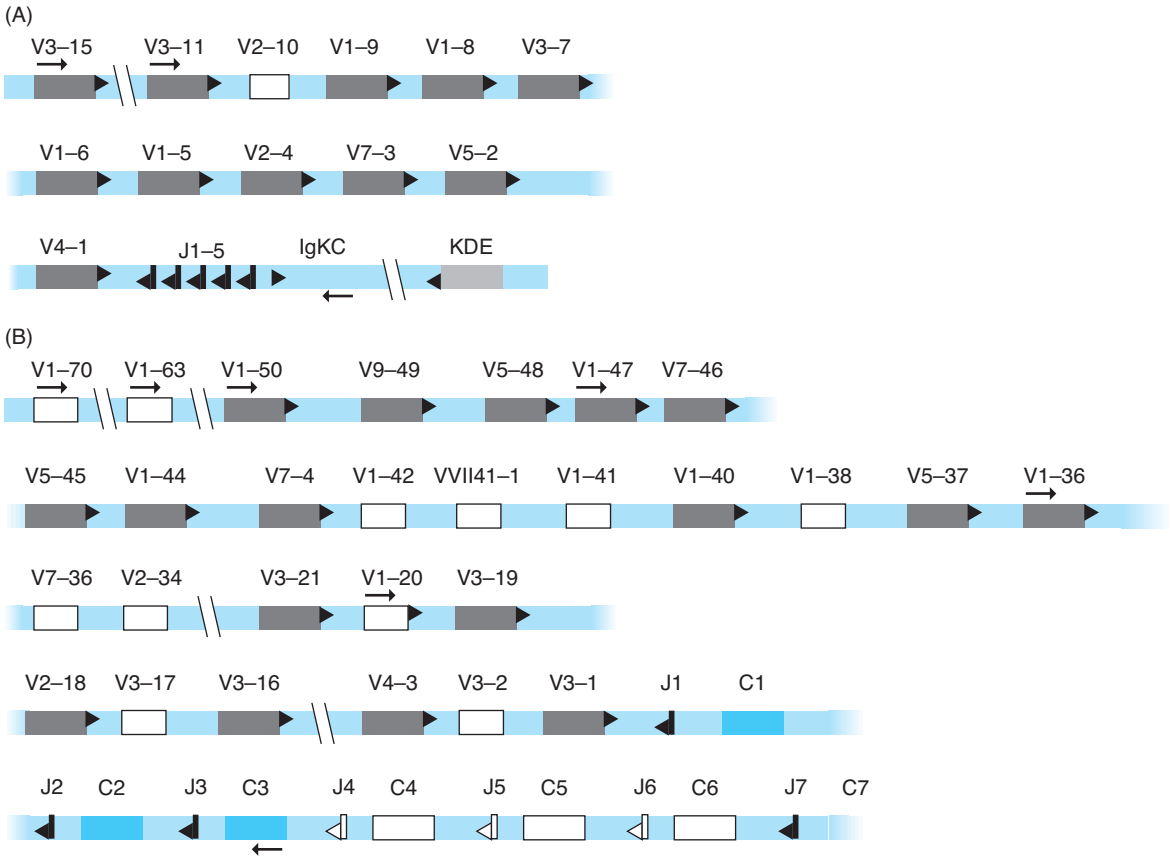


Figure 14-16 Immunoglobulin kappa (A) and lambda (B) light chain loci consist of gene segments for variable (V), joining (J), and constant (C) regions. The variable regions are classified into sequence-related families (V1-Vn). Each member of the family is given a number; for example, V7-4 is the fourth member of the V7 sequence family. Some of the gene segments are nonfunctional (open boxes). Recombination sites (triangles) are juxtaposed to each gene segment. Arrows denote primer binding sites for PCR clonality testing.

(V_λ) gene segments (29–33 functional) from 10 V_λ families and 7 J_λ (4–5 functional) and 7–11 C_λ gene segments (4–5 functional) occupying 1140-kb of DNA.[85]

Immunoglobulin light chain gene rearrangement is similar to that described for the immunoglobulin heavy chain gene. Selected gene segments are joined together with loss of the intervening DNA and possible insertion of nucleotides at the junction. The kappa locus rearranges first and then the lambda locus, if necessary. If the lambda locus rearranges, the kappa locus undergoes a secondary recombination through the KDE so that the cell does not produce both types of light chains.

During differentiation of the B cells from precursor stem cells, rearrangement, recombination, and mutation of the immunoglobulin V, D, and J regions ultimately results in functional VJ (light chain) and VDJ (heavy chain) genes.

T-Cell Receptor Gene Rearrangement

The T-cell receptor is composed of two of four chains, α, β, γ, and δ, with characteristic structures resembling immunoglobulin V, J and C regions (Fig. 14-17). The α and δ chains are encoded on chromosome 14 (the δ gene is located inside of the α gene), and γ and β are located

Advanced Concepts

Antibody diversity is ensured by three separate events during and after the gene rearrangement process. The first is the selection of gene segments. The second is the imprecise joining of the segments together with addition of nucleotide bases at the junction.[140] Third, after the gene rearrangement process has finished and the B cell has encountered antigen, **somatic hypermutation** occurs in the variable regions of the rearranged heavy and light chain genes. This mutation process requires the action of an enzyme called activation-induced cytidine deaminase, altering C residues to base pair with A instead of G residues, resulting in different amino acid substitutions in the antibody protein. Changing of the variable region sequences underlies the process of **affinity maturation**. As the B cells replicate, those producing antibodies with greater affinity to the antigen are favored, generating subclones of cells that may replace the original reactive clone. Over the course of an infection, therefore, antibodies with increased affinity are produced.

on chromosome 7. The four chains form pairs, making two types of receptors, αβ and γδ. T-cell receptor genes have fewer variable gene segments than the immunoglobulin genes, and the genes for the γ and α chains have no diversity regions (Table 14.6). The V regions of the receptor chains undergo gene rearrangement by intrachromosomal recombination as described for the immunoglobulin genes (Fig. 14-18).

Advanced Concepts

The T-cell receptor δ gene is flanked by TCR δ–deleting elements. Recombination between these elements or between Vα and Jα result in deletion of the TCRδ gene.

Rearrangement of the T-cell receptor chains proceeds in a similar manner as in the immunoglobulin genes. The V, (D), and J segments are joined together with addition or deletion (**trimming**) of nucleotides at the junctions between the gene segments.

The extracellular domains of the T-cell receptor dimers are held in conformation by interchain disulfide bridges between cysteine residues in the T-cell receptor peptides. The T-cell receptor chains also have a hydrophobic transmembrane region and a short cytoplasmic region. Although most cells express the αβ receptor, λδ receptors can represent a predominant population in certain tissues, such as the intestinal tract.

Detection of Clonality

Gene rearrangements occur independently in each lymphocyte so that a normal population of lymphocytes is **polyclonal** with respect to their rearranged immunoglobulin or T-cell receptor genes. Over-representation of a single rearrangement in a specimen cell population can be an indication of and characteristic for a lymphoma or leukemia. When over 1% of cells make the same gene rearrangement, the cell population is referred to as **monoclonal** with respect to the rearranged genes.

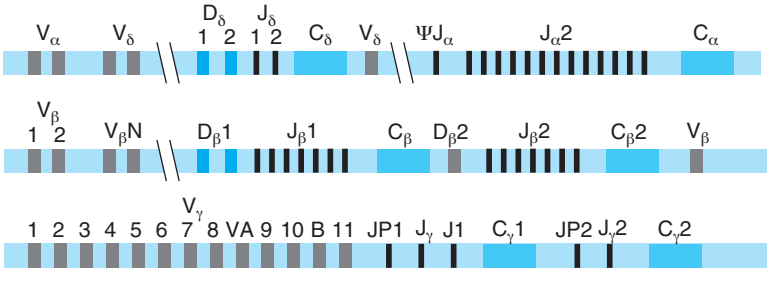


Figure 14-17 General structure of the T-cell receptor genes. The gene for the delta T-cell receptor chain is contained in the alpha locus (top). The beta and gamma chains are located at separate loci.

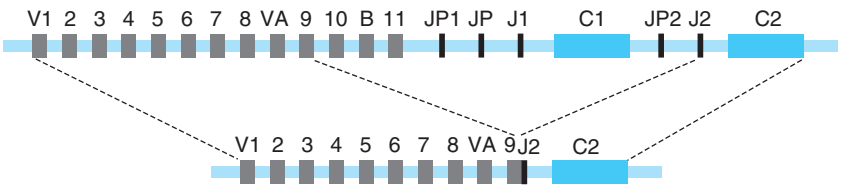


Figure 14-18 T-cell receptor gamma gene rearrangement occurs through selection of variable (V) and joining (J) segments.

Advanced Concepts

It is important to distinguish a large monoclonal population of tumor cells from a reactive clone or **oligoclone** or reactive clone of cells responding to an antigen. Oligoclones are not only smaller but transient in nature so that they should not be consistently present in serial analyses.

Immunoglobulin Heavy Chain Gene Rearrangements

Clonality can be detected by Southern blot.[86-88] With regard to the immunoglobulin heavy chain gene, restriction sites are mapped in the germline configuration. *Bam*H1, *Eco*R1, and *Hind*III are enzymes commonly used for this procedure (Fig. 14-19). When the germline sequence is rearranged, the restriction sites are moved, created, or deleted, resulting in a unique restriction pattern for every gene rearrangement. DNA cut with the restriction enzymes is transferred to a nitrocellulose membrane and probed to the joining region of the gene. Normal results should reveal the expected fragments generated from the germline DNA, in the example, an 18kbp *Eco*R1 fragment, an 18 kbp *Bam*H1 fragment, and an 11 kbp *Hind* III fragment. The normal fragments are visible for two reasons. First, a normal patient specimen contains cells other than lymphocytes that do not undergo gene rearrangement. The second reason for the presence of the germline bands is that only one chromosome in a lymphocyte undergoes gene rearrangement, leaving the homologous chromosome in the germline state. If the first rearrangement fails or is unproductive, then the second chromosome will rearrange. This occurs in fewer than 10% of lymphocytes for the heavy chain gene.

In a normal specimen, there will be millions of immunoglobulin gene rearrangements so that no one rearrangement is present in high enough amounts to be visible as non-germline bands on the membrane or autoradiogram; thus, only the germline bands will be visible. If, however, 2%–5% of the cells in the specimen consist of a clone of cells all with the same gene rearrangement, that clone will be detected by the presence of additional bands different from the germline bands (Fig. 14-20). Interpretation of the results is positive if extra bands are present and negative if only the germline bands are present.

Table 14.6 **T-Cell Receptor Gene Segments**

T-Cell Receptor Chain	Variable Gene Segments*	Diversity Gene Segments	Joining Gene Segments*	Constant Gene Segments
α	54/45	0	60/50	1
β	67/47	2	14/13	2
δ	3	3	4	2
γ	14/6	0	5	2

*Total gene segments/functional gene segments

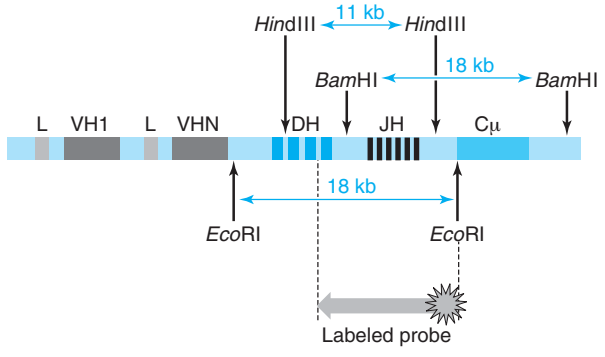


Figure 14-19 Restriction map of the germline immunoglobulin heavy chain gene. The fragments indicated by the arrows will be detectable with the probe shown at the bottom. Gene rearrangement will affect the placement of the restriction sites such that fragments of different sizes will be generated from a rearranged gene.

Advanced Concepts

Three enzymes are used for this assay to avoid false-positive results due to cross-hybridization artefacts (see Chapter 6). The chance of true rearranged bands being identical to cross-hybridization patterns for all three enzymes is negligible. In addition, cross-hybridization patterns are constant with the same hybridization conditions, in contrast to true monoclonal gene rearrangement bands that differ for each monoclonal population.

Analysis of clonality by Southern blot affords the advantage of detecting all gene rearrangements, including incomplete rearrangements involving only the D and J regions of the gene. The Southern blot method is limited by the requirement for at least 20–30 µg of high-quality DNA from the specimen. This is not always available, either because the specimen is limiting with respect to cell number or it is in poor condition as in paraffin-embedded specimens. Moreover, artifacts such as cross-hybridizations may complicate interpretation of Southern blot results.

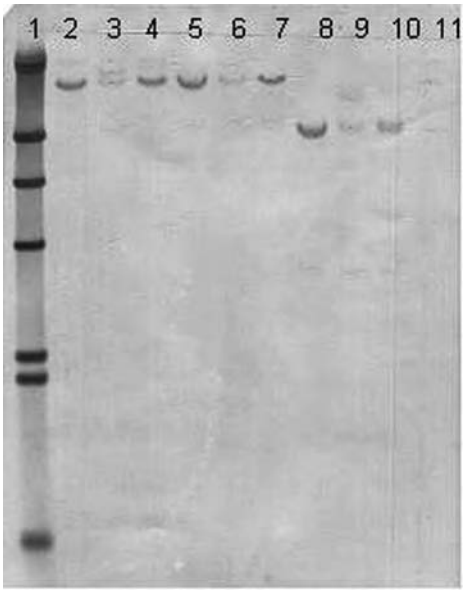


Figure 14-20 Colorimetric results from an immunoglobulin heavy chain gene rearrangement test by Southern blot with colorimetric detection. Lane 1, molecular weight markers. Lanes 2, 6, and 8 show the normal 18-kbp, 18-kbp, and 11-kbp bands expected from the germline gene in a normal specimen cut with *Eco*R1, *Bam*H1, and *Hind*III, respectively. Lanes 3, 6, and 9 show patient DNA with a monoclonal cell population. Lanes 4, 7, and 10 show a patient with no detectable monoclonality.

Gene rearrangement monoclonality at the immunoglobulin heavy chain gene locus is also analyzed by PCR. For this method, forward primers complementary to the variable region and reverse primers to the joining or constant regions of the B cell–rearranged genes are used.[89] The resulting amplicons are resolved by agarose, polyacrylamide or capillary electrophoresis. A normal cell population will yield amplicons consisting of fragments of different lengths, yielding a cloud or smear of bands. A monoclonal cell population will yield a predominant or single band. The presence of the band is interpreted as a positive result.

For immunoglobulin heavy chain gene rearrangement, different approaches to the variable region primer binding sites have been taken (Fig. 14-21). The immunoglobulin heavy chain gene variable region is divided into two types of domains. The **complementarity determining regions** (CDRs) code for the amino acids that will contact the antigen. The CDRs, therefore, are the most variable, or unstable, in sequence. The **framework regions** (FRs) code for the amino acids that have more of a structural role in the antibody protein and are more stable in

Advanced Concepts

The main limitation to detection of clonality by PCR is the loss of the FR3 primer binding site due to the gene rearrangement process that may destroy or remove the sequences bound by the FR3 primer. To address this issue, some laboratories use additional forward primers that bind to framework 2 (FR2) and framework 1 (FR1). Other methods utilize primers to the leader region in addition to the FR primers.[92,141,142] These primers increase the number of gene rearrangements that can be amplified and therefore detected by this assay. If the gene rearrangement cannot be amplified, clonality at the immunoglobulin heavy chain gene locus cannot be used for diagnosis or monitoring.

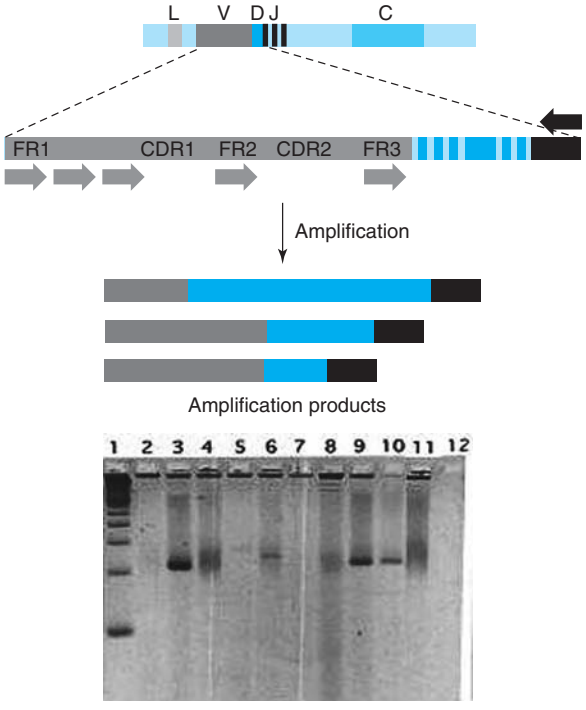


Figure 14-21 Immunglobulin heavy chain gene rearrangement by PCR with amplification from the variable region. Forward primers complementary to the variable region and reverse primers complementary to the joining region are used to amplify the diversity region (top). In a polyclonal specimen, amplification products in a range of sizes will result. These products produce a dispersed pattern on an ethidium bromide–stained agarose gel (lanes 4, 8, 11). If at least 1% of the sample is representative of a monoclonal gene rearrangement, that product will be amplified preferentially and revealed as a sharp band by gel electrophoresis (lanes 3, 6, 9, and 10).

sequence. Standard methods for clonality utilize a forward **consensus primer** to the innermost framework region (FR3) and the reverse primer complementary to the joining region. The consensus primer has sequences that match the most frequently occurring sequences in the FR3 region and may not be identical to any one sequence. Enough nucleotides will hydrogen-bond, however, to match most rearranged variable regions.

Primers directed at the diversity region are useful for amplification of the germline configuration of the immunoglobulin heavy chain genes as well as D_H-J_H rearranged genes.[90] Primers complementary to the 7 family-specific sequences of the diversity region and a primer to the

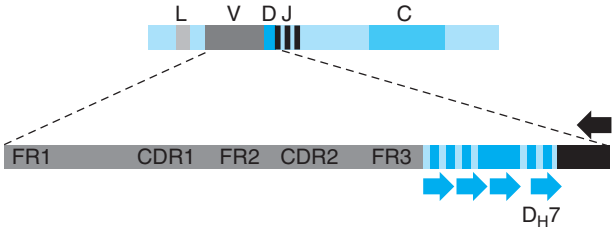


Figure 14-22 Immunglobulin heavy chain gene rearrangement by PCR with amplification from the diversity region. Forward primers complementary to the diversity region and reverse primers complementary to the joining region yield a polyclonal pattern in normal samples. The D_H7 primer will yield a specific 350-bp product from the unrearranged (germline) gene due to the short distance between the D_H7 gene segment and the joining region primer.

5'-most joining region (J_H1) are used to amplify the D_H-J_H junction. The primer complementary to the D_H7-27 segment, the sequences closest to the joining region, will yield a defined product from the Ig heavy chain gene in the germline configuration (Fig. 14-22). Diversity region primers are also useful for targeting incomplete rearrangements where the variable region primer binding sites are lost[91,92]

Another approach to the detection of B-cell clonality is to make patient-specific primers. For this method, a consensus primer is used to amplify the rearranged gene from a positive specimen. The amplification product is then purified from the gel or from the PCR reaction mix and sequenced. Primers exactly matching the variable region sequence of that specimen are then manufactured for use on subsequent samples. The advantage of this method is that it is more sensitive, as fewer or none of the gene rearrangements from normal cells are amplified. Furthermore, the tumor load can be measured quantitatively by real-time PCR. The disadvantage of this approach is that the method is more time-consuming to perform, and the primers used are patient-specific. Moreover, in a patient with a chronic condition, for whom monitoring is likely to be done, tumors cells may undergo mutation in the variable region and inactivate the specific primer binding, requiring manufacture of new primers.

Immunoglobulin Light Chain Gene Rearrangements

The immunoglobulin light chain genes are also targets for clonality detection.[93–95] In addition to Southern blot, PCR

and RT-PCR have been used to detect light chain gene clonality. Targeting the immunoglobulin light chain genes is especially useful for tumors arising from terminally differentiated B cells (plasma cells) that have undergone extensive somatic hypermutation at the heavy chain gene locus. In these tumors, the rearranged heavy chain genes are frequently unamplifiable, yielding false-negative results because of accumulation of base changes in the variable region primer binding sites. Most of these tumors are amplifiable, however, at the light chain genes.[96]

Gene rearrangements in the kappa light chain locus are amplified using primers complementary to the sequence families of the V_κ region or to the intron between the J_κ regions and C_κ. Opposing primers are complementary to C_κ and to one or more of the five joining regions. Alternately, the KDE can be used for a primer binding site (Fig. 14-23). Using the KDE allows detection of lambda-expressing cells that have deleted J_κ or C_κ.[97]

Approximately one-third of B-cell malignancies have Ig_λ gene rearrangements.[98] Ig_λ gene rearrangements are also present in 5%–10% of kappa-expressing B-cell tumors. Therefore, detection of clonality at the Ig_λ locus on chromosome 22 is also useful for confirming or monitoring diagnosis of B-cell leukemias and lymphomas. Forward primers complementary to V_λ gene segments and reverse primers to the J_λ and C_λ gene segments are frequently used for these assays (Fig. 14-24).

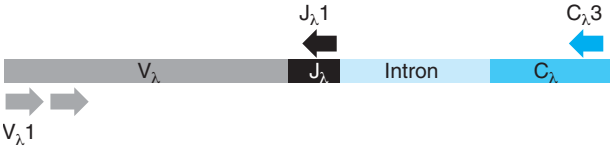


Figure 14-24 The immunoglobulin light chain lambda locus can be amplified by standard PCR procedures from the variable region to the joining region or the constant region. Reverse transcriptase PCR is used with the constant region primers to eliminate the intron between the constant and joining regions.

T-Cell Receptor Gene Rearrangements

T-cell receptor gene rearrangements are performed in a manner similar to the immunoglobulin gene rearrangements by Southern blot and PCR.[99–101] For Southern blot studies, probes complementary to the variable, joining, and diversity regions of the T-cell receptor genes are used to detect monoclonal populations. Just as with immunoglobulin gene rearrangements, no one gene rearrangement should be visible in a normal specimen. The presence of a large monoclonal population is revealed by the bands detectable in addition to the germline bands seen in the normal control (Fig. 14-25).

The T-cell receptor gene rearrangement assays done by PCR target most often the TCRγ gene. Assays are also performed on the TCRβ and TCRδ genes. Detection of gene rearrangements in TCRα is difficult due to the 85-kb length of the Jα gene segments. TCRα gene rearrangements may be inferred from TCRδ gene deletions.[101] Primers are designed complementary to the rearranged gene segments (Fig. 14-26). Multiple primer sets are often used to ensure detection of the maximum potential gene rearrangement.[102] PCR will yield a pro-

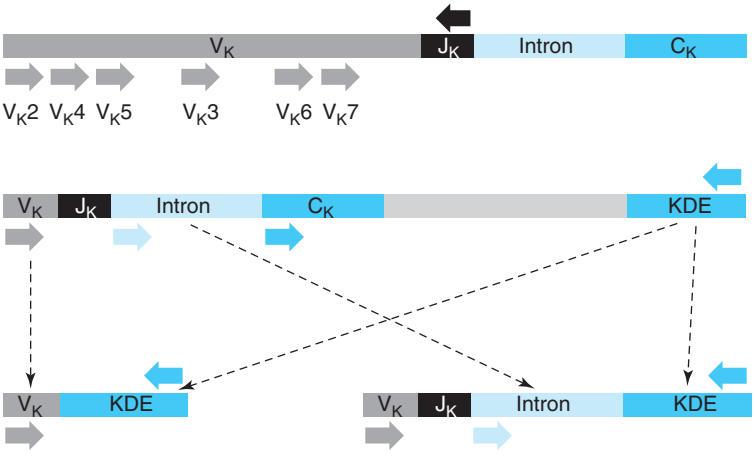


Figure 14-23 The immunoglobulin light chain kappa locus can be amplified by standard PCR procedures from the variable region, the intronic region, or the kappa deleting element.

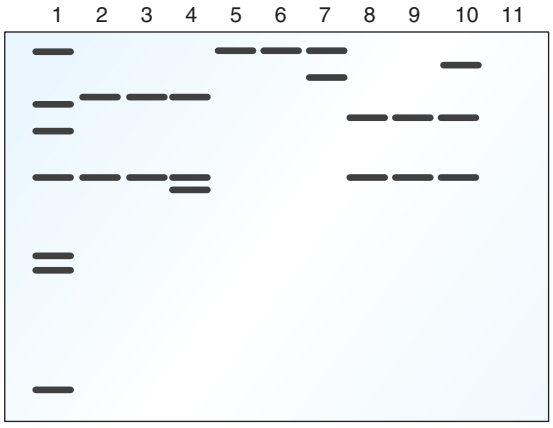


Figure 14-25 T-cell receptor gene rearrangements detected by Southern blot using a probe mixture of sequences complementary to $J_\beta 1$and $J_\beta 2$. Lane 1, molecular weight markers. Lanes 2, 5, and 8, normal control cut with *Eco*R1, *Bam*H1, and *Hind*III, respectively. Lanes 3, 6, and 9, a negative specimen cut with *Eco*R1, *Bam*H1, and *Hind*III, respectively. Lanes 4, 7, and 10, positive specimen cut with *Eco*R1, *Bam*H1, and *Hind*III, respectively. Note the additional bands in the positive specimen lanes.

duct consistent with a single gene rearrangement in a positive sample, whereas a normal sample will yield a polyclonal pattern (Fig. 14-27). Due to the limited range of lengths of the population of rearranged T-cell receptor genes, heteroduplex analyis, SSCP, or DGGE may be

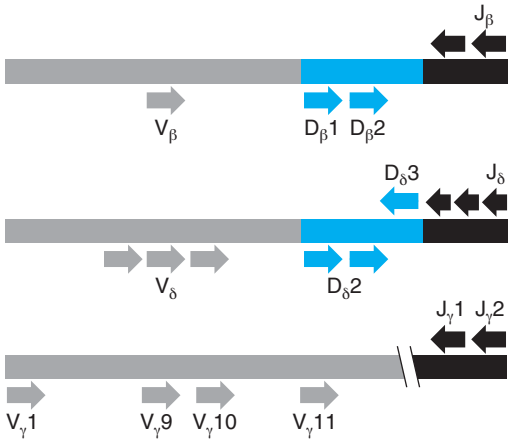


Figure 14-26 T-cell receptor gene rearrangements detected by PCR use primers to the variable, diversity, and joining regions of the rearranged genes.

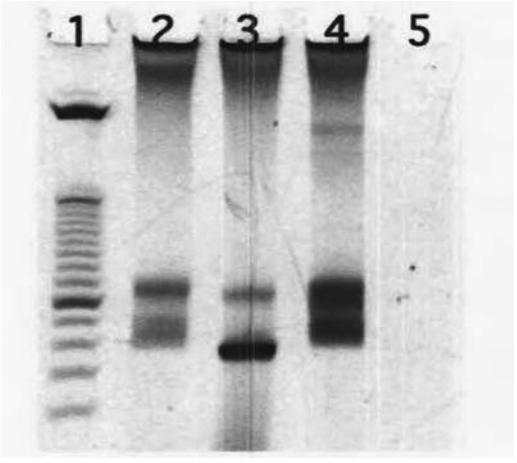


Figure 14-27 T-cell receptor gene rearrangement by PCR and heteroduplex analysis. Bands were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. Lanes 2 and 4 show a polyclonal pattern. A positive result (monoclonal pattern) is shown in lane 3. Lane 1, molecular weight marker; lane 5 reagent blank.

used to improve resolution of the polyclonal and monoclonal patterns.[103]

Banding Patterns

Interpretation of clonality by PCR depends on gel banding patterns. False-negative results may arise from primers that do not match the gene rearrangement in the tumer. False-positive results arise from artifactual single bands. Patterns of multiple single bands may arise from specimens with low cell numbers, such as cerebrospinal fluids or paraffin sections. In this case, usually more than one or two single bands occur. The bands may not indicate monoclonality, rather that only a few cells are present. These cells may or may not be malignant. False single band patterns may also occur within polyclonal smears detected by nondenaturing polyacrylamide gels (PAGEs). Heteroduplex analysis is recommended if amplicons are resolved by nondenaturing PAGEs.

Similar problems may arise with resolution by capillary electrophoresis where peak heights and widths are compared to distinguish wide polyclonal peaks from narrow monoclonal spikes.[104] Pretreatments such as eosin staining may yield false single bands by capillary electrophoresis.[105]

Translocations in Hematological Malignancies

Gene rearrangements in immunoglobulin and T-cell receptor genes are normal and take place regardless of the presence of malignancy. The unique nature of the gene rearrangement system with cell-specific antibody and antibody receptor formation are exploited in finding abnormal cell populations clonally derived from these cell-specific features. Targeting these gene rearrangements, however, can only be applied to tumors arising from lymphocytes. Other types of malignancies, such as myeloid tumors, cannot be analyzed using these targets.

Often, development of the cancer state results in DNA anomalies, such as translocations or other types of mutations, that can be applied to clinical discovery and monitoring of tumors. These abnormal genetic events aid in diagnosis of specific types of hematological tumors, such as the translocations that are highly associated with chronic myelogenous leukemia, promyelocytic leukemia, or follicular lymphoma. Mutations or disruptions of expression of tumor suppressor genes and oncogenes are usually involved in hematological malignancies, which has helped to determine the molecular events leading to the tumor phenotype.

Translocations are the exchange of DNA between chromosomes (see Chapter 8). If the translocation disrupts activity or expression of oncogenes or tumor suppressor genes, a cancer phenotype can occur. Several translocations are frequently found in certain types of tumors.

- t(14;18)(q32;q21)

The reciprocal translocation between the long arms of chromosomes 14 and 18 moves and disregulates the *BCL2* gene located on chromosome 18q21.3. *BCL2* (B-cell leukemia and lymphoma 2 or B-cell CLL/lymphoma 2) is an oncogene. The gene product of *BCL2* is a member of a group of related proteins that control apoptosis (cell death initiated by internal cellular signals). The Bcl2 protein inhibits apoptosis in B lymphocytes, that is, enhances survival of cells that normally would die. Survival of genetically damaged cells may contribute to the development of tumors. One of the most frequent hematological malignancies, follicular lymphoma, is associated with the t(14;18) translocation.

The translocation partner of chromosome 18 is chromosome 14 in the vicinity of the immunoglobulin heavy chain gene. The translocation may occur through cryptic recognition sites for the gene rearrangement recombinase enzymes on chromsome 18. As a result, an abnormal interchromosomal exchange occurs, instead of the normal intrachromosomal exchange events described in the previous section (Fig. 14-28). There are several breakpoints in the chromosome 18 region 3' to the *BCL2* gene, most of which occur in the **major breakpoint region** (MBR). About 10%–20% of the breakpoints fall into a cluster closer to the BCL2 gene, thousands of bases from the MBR, in the **minor cluster region** (MCR). An intermediate cluster region (ICR) and other breakpoints outside of MBR and MCR have also been reported.[106,107]

Molecular detection of the t(14;18) translocation is performed by Southern blot or PCR. For Southern blot, a probe to the MBR region of chromosome 18 will reveal the translocation by the presence of bands different from those expected from normal chromosome 18 (Fig. 14-29). The Southern blot procedure has the advantage of detecting all breakpoints. The translocation is more easily and rapidly detected by PCR. Forward primers to chromosome 18 and reverse primers complementary to the Ig heavy chain joining region will yield a product only if the two chromosomes have been joined by the translocation. The amplicons are visualized by gel electrophoresis as shown in Figure 14-30 or by capillary electrophoresis, the latter method requiring fluorescent labeling of one of the PCR primers.

The t(14;18) translocation can also be detected using real-time PCR. Several methods are available for this analysis, and although laboratory methods differ, results are reasonably consistent in comparison testing.[108,109]

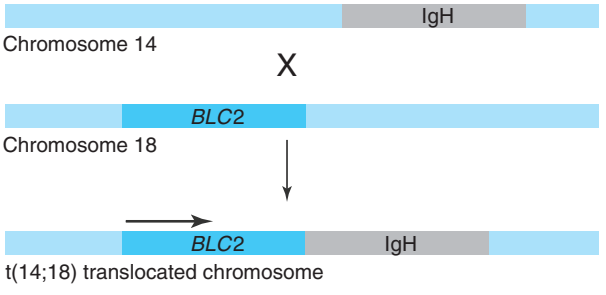
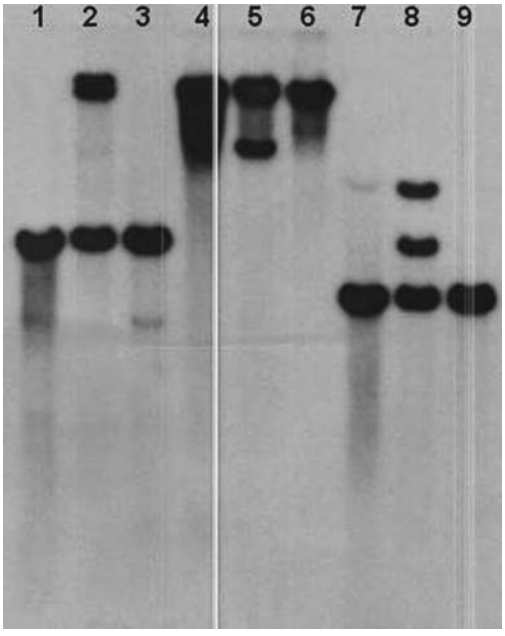


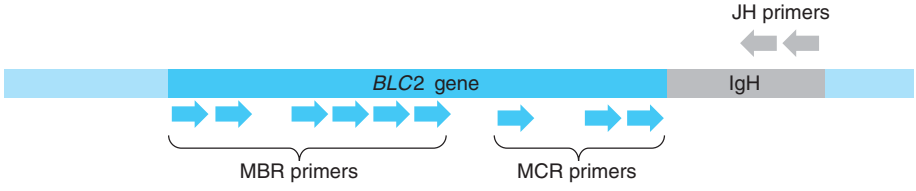
Figure 14-28 The t(14;18) translocation moves the *BCL2* gene intact to the long arm of chromosome 14 next to the joining region of the immunoglobulin heavy chain gene (IgH). The translocation breakpoints on chromosome 18 are 3' to the *BCL2* gene (arrow indicating the direction of transcription).

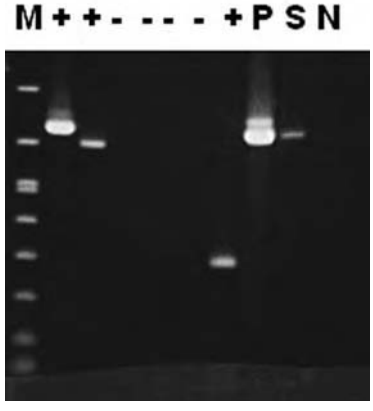


■ Figure 14-29 Analysis of the t(14;18) gene translocation by Southern blot with chemiluminescent detection. Lanes 3, 6, and 9 are the normal control cut with *Eco*R1, *Bam*H1, and *Hind*III, respectively. Lanes 1, 4, and 7 show results from a normal patient specimen. Lanes 2, 5, and 8 are results from a specimen positive for the t(14;18) translocation. Note the extra bands in the positive specimen.

Most methods include a standard curve for regression analysis of the test sample measurements (Fig. 14-31). Alternatively, internal controls, such as known amounts of plasmid DNA, may be added to the test specimens. In this method, the amount of translocated cells (or translocated chromosomes) is determined relative to the internal control. There are several approaches to reporting final results. Most frequently, results are reported as percent translocated cells in the specimen tested. For instance, the raw number of translocated cells is determined by linear regression analysis (using the formula $y = mx + b$). The raw number of cells is then divided by the number of total cells represented in the PCR reaction.

For example: for a dilution series ranging 50–10,000 translocated cells in 1 million untranslocated cells, the standard curve generates the formula $y = 1.5631\text{Ln}(x) + 42.396$, where y is the threshold cycle number (see Chapter 7) and x is the number of translocated cells (50 ng of DNA was used per sample). Using this formula, if a given sample crosses the fluorescent threshold at $y = 36$ cycles (average of duplicate measurements), the number of cells (x) is approximately 60 cells. Assuming that 50 ng of DNA represents 7500 cells (1 ng of DNA = approximately 150 cells), $60/7500 = 0.008$. $0.008 \times 100 = 0.8\%$ translocated cells in the specimen. For the t(14;18) translocation, sensitivities of 0.0025% have been





■ Figure 14-30 Analysis of the t(14;18) gene translocation by PCR with agarose gel electrophoresis. Several primers are used for detection of the translocated chromosome (top). Because the forward primers are on chromosome 18 and the reverse primers are on chromosome 14, a PCR product will be generated only from the t(14;18) translocated chromosome (bottom). M, molecular weight marker; +, positive specimen; -, negative specimen; P, positive control; S, sensitivity control; N, negative control.

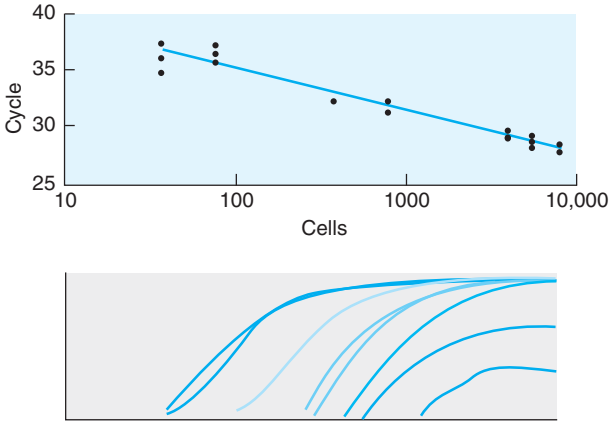


Figure 14-31 t(14;18) translocation analysis by real-time PCR. A standard curve is established using cultured cells with the t(13;18) translocation (Dhl-6) counted and diluted into cultured cells without the translocation (HL-60). The results shown are from DNA isolated from each mixture of cells and analyzed by real-time PCR using a TaqMan probe.

reported, with a linear range of 0.01%–10%.[110] Data are accumulating to determine the clinical significance of the qualitative and quantitative results.[111–113]

The main limitation of any PCR procedure targeting the t(14;18) translocation is the inability of the primers to detect all of the possible breakpoints on chromosome 18. Several primer pairs and sets of primer pairs have been designed to address this problem.[89] Test reports should include an estimation of false-negative results expected due to breakpoints that remove the binding sites for the primers used. Unless a translocation has been previously observed by a given PCR method, a negative result may be reported as indeterminate, acknowledging that a translocation may be present but undetectable with the primers used.

- t(9;22)(q34;q11)

The t(9;22) translocation is a reciprocal exchange between the long arms of chromosomes 9 and 22. The translocation generates the Philadelphia chromosome (Ph1), which is present in 95% of cases of chronic myelogenous leukemia (CML), 25%–30% of adult acute lymphoblastic leukemia (ALL), and 2%–10% of pediatric ALL. The breakpoints of the t(9;22) translocation occur within two genes, the breakpoint cluster region (*BCR*) gene on chromosome 22 and the cellular counterpart of the Abelson leukemia virus tyrosine kinase (c-*abl*) on chromosome 9 (Fig. 14-32). The result of the translocation is a chimeric or **fusion gene** with the head of the *BCR* gene and the tail of the c-*abl* gene. Both genes are tyrosine kinases; that is, they phosphorylate other genes at tyrosine residues. The fusion gene is also a kinase but has aberrant kinase activity.

There are two major forms of the BCR/ABL fusion gene, joining either exon 13 or 14 (b2 or b3) of the BCR gene to c-*abl* exon 2 (a2; Figure 14-33). The b2a2 or b3a2 fusion genes code for a 210 kilodalton protein,

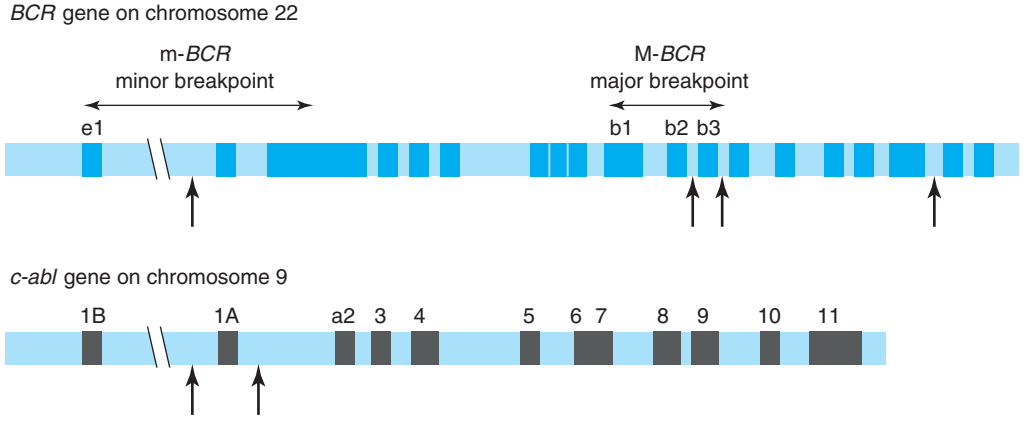


Figure 14-32 The t(9;22) translocation begins with breakage of chromosomes 9 and 22 in introns of the BCR and c-*abl* genes (arrows). The breakpoints are located within introns of both genes.

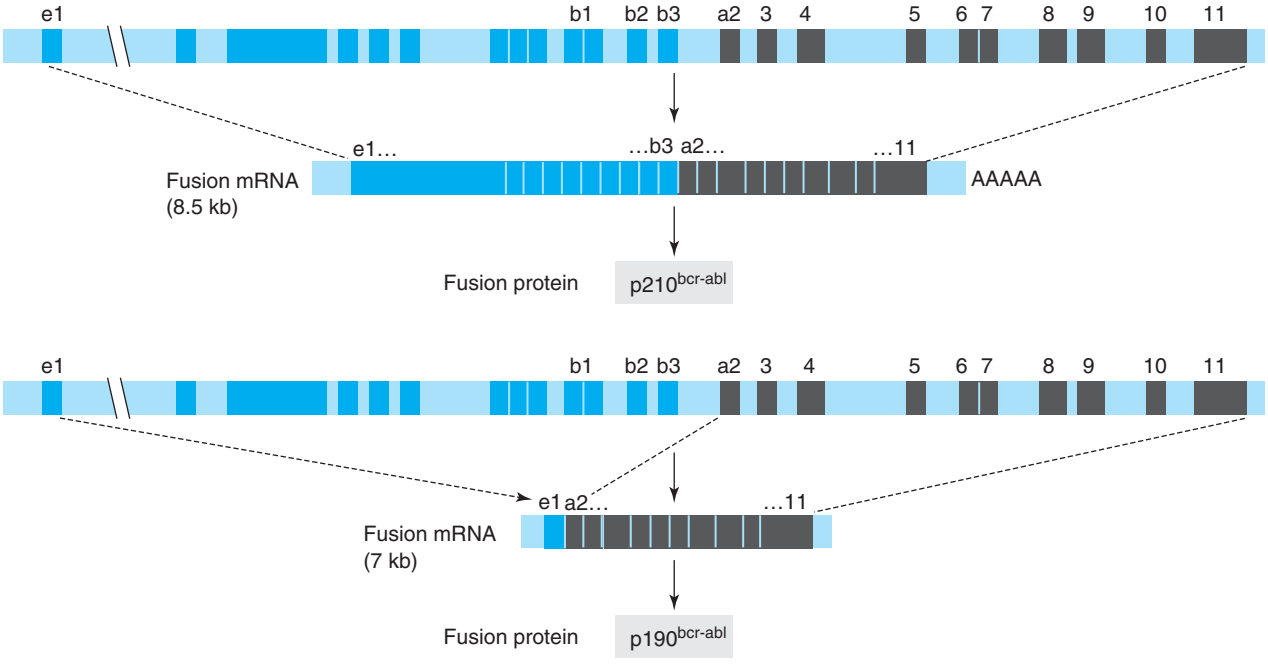


Figure 14-33 p210 and p190 are the two main fusion proteins produced by the t(9;22) translocation. They differ in the amount of the *BCR* gene that is attached to the c-*abl* gene.

p210. A third form of the fusion gene joins exon 1 of *BCR* with exon a2 of c-*abl*, resulting in expression of an e1a2 transcript, which codes for a p190 protein. Another, less common, fusion junction occurs at exon 19 of the BCR gene (c3). The c3a2 transcript encodes a p230 protein. All of the fusion proteins have been observed in CML; however, p190 occurs mostly in ALL.

Detection of the t(9;22) translocation can be performed by Southern blot in a manner similar to that described above for detection of t(14;18). One probe used for this test is a 1.2-kb fragment complementary to the 3' end of the *BCR* gene. Restriction fragment patterns will differ in translocated cells (Fig. 14-34). Other probes to BCR may also be used to ensure detection of all breakpoints.

The common rationale for detection of translocations by PCR is used for t(9;22); that is, forward primers are designed to hybridize to the *BCR* gene on chromosome 22 and reverse primers to chromosome 9 in the c-*abl* gene (Fig. 14-35). A product will result only if the two genes are joined by the translocation. Due to the length of the introns that separate the primer binding sites, nested RT-

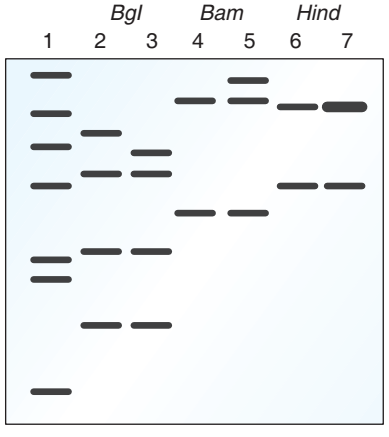


Figure 14-34 Southern blot of a t(9;22) translocation with a probe to the 3' end of the *BCR* gene. Lane 1, molecular weight marker; lanes 2, 4, and 6, normal control cut with *Bgl*II, *Bam*H1, and *Hind*III, respectively; lanes 3, 5, and 7, a specimen positive for the translocation cut with *Bgl*II, *Bam*H1, and *Hind*III, respectively. Note the shift in the sizes of the bands in lanes 3 and 5 compared with lanes 2 and 4. (A doublet is not quite resolved from the larger band in lane 7.)

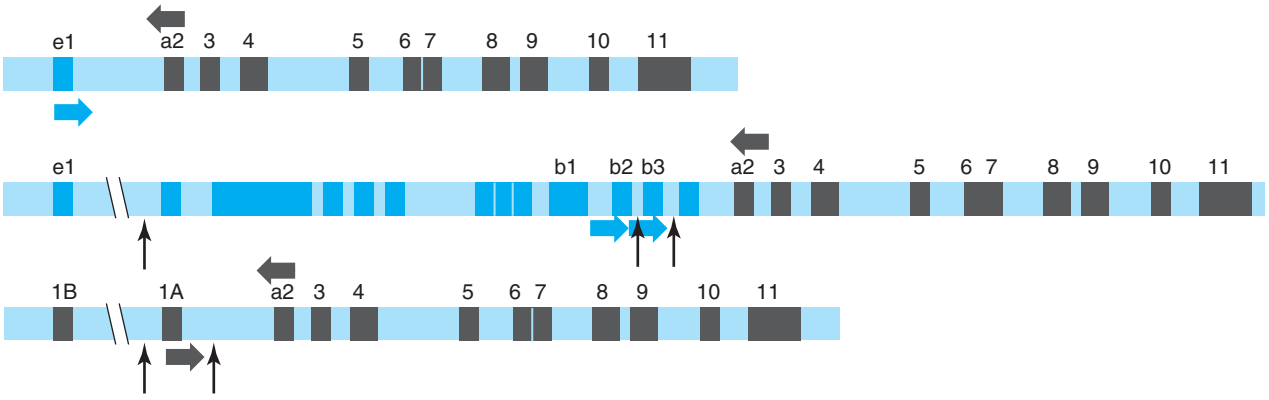


Figure 14-35 Location of primers (horizontal arrows) for PCR analysis of the minor breakpoint region (top) and the major breakpoint region (middle). The c-*abl* gene itself may be used as an amplification control (bottom). The intron and exon lengths are not drawn to scale. Vertical arrows denote locations of breakpoints.

PCR has been commonly utilized.[114,115] To minimize the risk of false-positive results, this method may require confirmatory testing, especially for adult ALL.[116] Several recommendations for optimal performance have been proposed, including the use of primers capable of detecting both major and minor breakpoints in the BCR gene and an internal **RNA integrity control** (or amplification control) to avoid false-negative results from poor RNA quality or inadequate cDNA synthesis.[117–118] Transcripts from the *abl* or *BCR* genes are used most frequently for the RNA integrity control for the t(9;22) translocation, but any unique gene with constitutive expression may be used. Target amplicons can be detected by agarose gel electrophoresis and ethidium bromide staining (Fig. 14-36) or by capillary gel electrophoresis. Fluorescent dye–labeled primers are required for the latter detection method.

Real-time PCR provides quantitative estimation of treatment response, especially with novel therapies for CML and ALL. Although cytogenetic methods, espe-

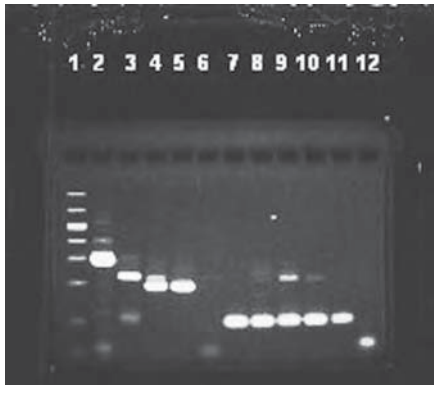


Figure 14-36 Results of a standard RT-PCR test for the t(9;22) translocation. Lane 1, molecular weight marker; lane 2, b3a2 breakpoints; lane 3, b2a2 breakpoints; lanes 4 and 5, e1a2 breakpoints; lane 6, negative specimen; lanes 7–11 are RNA integrity (amplification) controls for specimens in lanes 2–6; lane 12, reagent blank.

cially FISH and standard RT-PCR, are most practical for diagnosis and in the early stages of treatment, quantitative PCR provides a valuable estimation of tumor load over the course of treatment.[119,120]

Because transcript levels are being measured using RT-PCR, it is important to stabilize the specimen RNA on receipt, for example by resuspending the white blood cells in protective buffers (see Chapter 4). Another recommendation is to collect at least 10 mL of peripheral blood for analysis to avoid false-negative results.[121] Primers used for this method are similar to those used for standard PCR. A TaqMan probe provides the fluorescent signal. A standard curve or a high and low positive control and negative control should accompany each run. Frequently used TaqMan methods report measurements as a ratio of the *BCR-abl* transcript level to the RNA integrity control, usually the *abl* transcript, the *BCR* transcript, or the transcript of a housekeeping gene, such as G6PDH[119,122–124] For example, a standard curve for transcript number (Fig. 14-37) generates the formula, $y = -1.7318\text{Ln}(x) + 48.627$, where y is the threshold cycle number and x is the number of transcripts. If quantitative PCR analysis of the patient specimen RNA yielded a threshold cycle number of 39 (average of duplicate samples) for *BCR-abl* transcripts and a threshold cycle number of 30 for the *abl* transcripts, then solving for x yields 300 *BCR-abl* transcripts and 50,000 *abl* transcripts in the sample. Thus, $(300/50{,}000) \times 100 = 0.6\%$.

Data are still being collected with regard to the clinical significance of the quantitative results. A three-log drop in transcript levels or a *BCR-abl/abl* × 100 level below 0.05% have been proposed as indicators of good prognosis.[125 126]

- t(15;17)(q22;q11.2-q12)

Advanced Concepts

A slightly different formula, [*BCR-abl/(abl - BCR-abl)*] × 100, for the transcript ratio is used if the *abl* primers also amplify the *BCR-abl* translocation.[143] In the calculation shown in the text, *BCR-abl/abl* × 100 yields 0.6%; [*BCR-abl/(abl - BCR-abl)*] × 100 yields 0.85%.

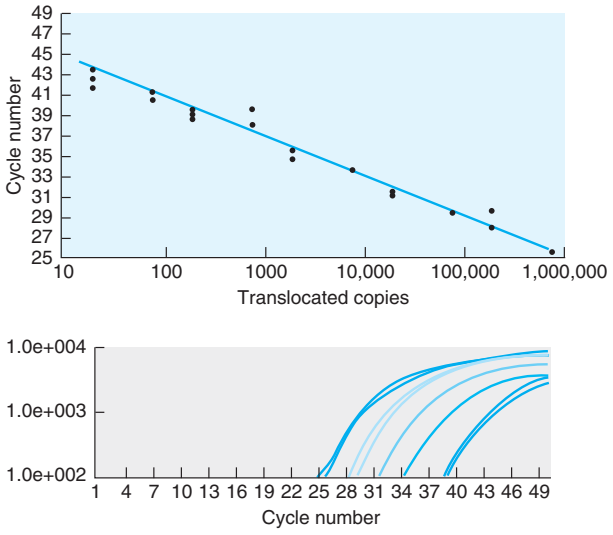


Figure 14-37 Real-time PCR analysis of transcript levels requires a standard curve of known transcript numbers. The number of translocated transcripts in the specimen can then be determined from the standard curve.

This reciprocal translocation between the long arms of chromosomes 15 and 17 results in fusion of the retinoic acid receptor alpha (*RARA*) gene on chromosome 15 with the myelocytic leukemia (*MYL*, or *PML*) gene on chromosome 17. Both genes contain zinc finger binding motifs and therefore bind DNA as transcription factors. The *PML/RARA* fusion is found specifically in promyelocytic leukemia. The presence of this translocation is also a predictor of the response to retinoic acid therapy that is used as treatment for this disease. The translocation forms a fusion gene with the first three (type A or S translocation) or six (type B or L translocation) exons of the *PML* gene joining to exons 2–6 of the *RARA* gene (Fig. 14-38).

Test methods similar to those described above for *BCR-abl* translocation are used to detect t(15;17). Reverse transcriptase PCR and real-time reverse transcriptase PCR are most frequently used. Primers complementary to sequences in exon 3 or 6 of the *PML* gene and exon 2 of *RARA* generate products only if the translocation has occurred. The presence of the translocation product, therefore, is interpreted as a positive result. As with any test of this type, an amplification control is required to avoid false-negative results. For quantitative PCR, results normalized to an internal control, using calcula-

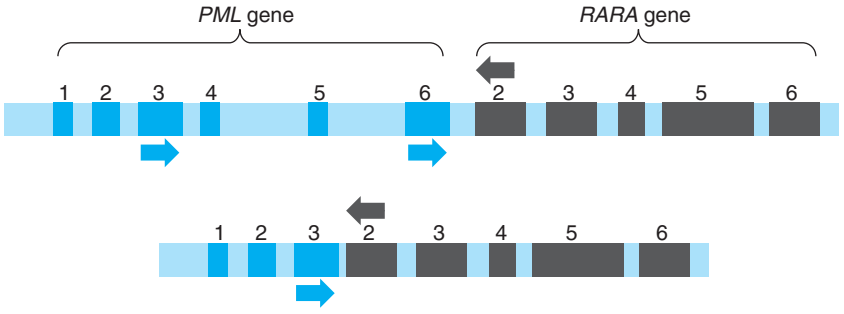


Figure 14-38 Location of primers for PCR analysis of the long (top) and the short (bottom) translocated genes. The first three or six exons of the *PML* gene is fused to exon 2 of the *RARA* gene. The intron and exon lengths are not drawn to scale.

tions as described above, yield the most consistent day-to-day results (lowest coefficient of variance).[127]

- t(11;14)(q13;q32)

This translocation joins the immunoglobulin heavy chain gene region on chromosome 14 with part of the long arm of chromosome 11. The cyclin D1 (*CCND1*) gene, also called the parathyroid adenomatosis 1 gene (*PRAD 1*) or *BCL1*, on chromosome 11 is attached to the long arm of chromosome 14 in the intron between the immunoglobulin heavy chain gene joining and constant regions. The translocation increases expression of *CCND*1, resulting in passage of the cell cycle from the G1 to the S phase of the cell cycle. This translocation is found primarily in mantle cell lymphoma (MCL) but may also be present in chronic lymphocytic leukemia, B-prolymphocytic leukemia, plasma cell leukemia, multiple myeloma, and splenic lymphoma. The t(11;14) translocation is thought to be a definite characteristic of MCL; however, only 50%–70% of MCLs have a detectable t(11;14).

Methods for detection of t(11;14) are similar to those used for t(14;18) translocation described above. Southern blot methods have been replaced mostly with PCR and RT-PCR.[128] In situ hybridization to detect an increase in CCDN1 transcription has also been proposed.[129] Of the breakpoints on chromosome 11, 80% are in the major translocation cluster 5′ to the *CCND*1 gene (Fig. 14-39). The rest are dispersed in other areas 5′ or 3′ of the gene. PCR analysis detects 40%–60% of the translocation breakpoints.[130] A PCR product will result only if the translocation has occurred (and the primer binding

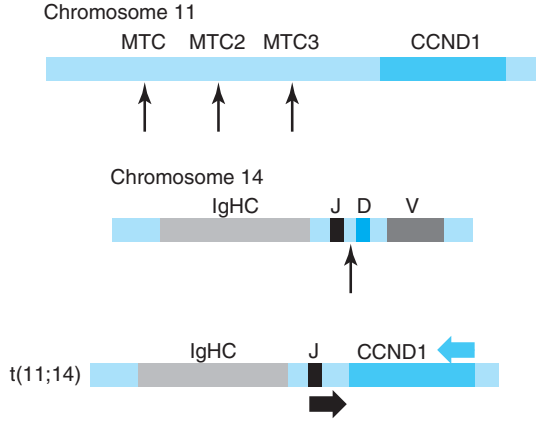


Figure 14-39 The t(11;14) breakpoints in chromosome 11 (top) and chromosome 14 (center) are indicated by the vertical arrows. Primers are complementary to the joining region of the immunoglobulin heavy chain gene and the *CCND1* gene (bottom).

Advanced Concepts

In this translocation, the reciprocal fusion gene *RARA/PML* is also expressed in 70%–80% of cases of APL. Nonreciprocal events can produce either fusion alone as well.[144,145] Although the reciprocal fusion may participate in the tumorigenesis process, it does not predict response to all transretinoic acid therapy.[146] The presence of the reverse transcript may be used to confirm the translocation and could be useful in cases where the *PML/RARA* transcript is poorly expressed.

Advanced Concepts

A variety of molecular events can result in the overexpression of *c-myc*. The DNA virus Epstein Barr virus (EBV) is associated with Burkitt's lymphoma.[147] EBV induces cell proliferation and, thus, provides more opportunity for translocation events. Certain types of another DNA virus, human papillomavirus, inserted into the vicinity of *c-myc* cause overexpression of the gene.[148] Transposable elements such as LINE-1 elements may also destabilize oncogenes.[149]

sites are maintained). The PCR product can be detected by gel or capillary electrophoresis.

- t(8;14)(q24;q11)

The avian myelocytomatosis viral oncogene homolog (*c-myc*) gene on chromosome 8 is one member of a gene family including *n-myc* and *l-myc*. The *c-myc* gene codes for a helix-loop-helix/leucine zipper transcription factor that binds to another protein, Max, and activates transcription of other genes. The t(8;14) is associated with Burkitt's lymphoma; in addition, translocations at (2;8) and t(8;22) are found in about 10% of Burkitt's lymphomas.

In the t(8;14) translocation, the breakpoints on chromosome 8 are spread over a 190-kbp region 5' to and within the *c-myc* gene. As a result of the t(8;14) translocation, the *c-myc* gene is separated from its normal promoter and regulatory region and moved into the switch recombination region of the immunoglobulin heavy chain gene on chromosome 14. In the t(2;8) and the t(8;22) translocations, the chromosome 8 breakpoints are 3' to the gene, and *c-myc* is moved into the immunglobulin kappa or lambda locus, respectively. Translocations of *c-myc* into the T-cell receptor alpha gene have also been reported.[131]

In the laboratory, *c-myc* translocations are commonly detected by Southern blot analysis with a probe complementary to exon 3 of the *c-myc* gene; for instance, a ^{32}P- or digoxigenin-labeled 1.4-kb *Cla*I-*Eco*RI restriction fragment. Interphase FISH and CISH (Invitrogen) can

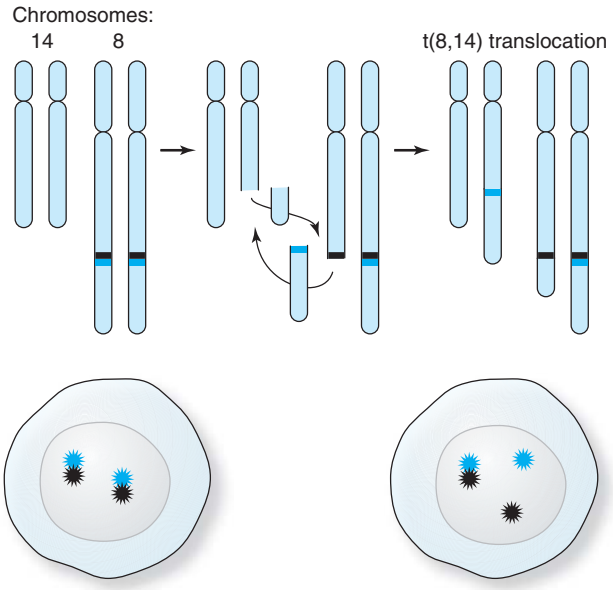


Figure 14-40 The t(8:14) breakpoint detected by CISH (Invitrogen). Two probes labeled with biotin or digoxigenin are complementary to sequences flanking the chromosome 14 breakpoint. In the absence of the translocation (left), the probes will appear next to one another in the nucleus. The translocation (right) will move one probe to chromosome 14, leaving the other behind on chromosome 8, resulting in separation of the signals in the nucleus.

also be used to detect the t(8;14) translocation with separate probes to chromosomes 8 and 14 or with chromosome 14 probe pairs (Fig. 14-40).[132] Overexpression of *c-myc* is detectable by Northern blot.[133] Amplification of the gene can be detected by FISH using a 120-kb *c-myc* (8q24.12-q24.13) probe labeled with SpectrumOrange (Vysis).[134]

- FMS-related Tyrosine Kinase 3 (FLT3), 13q12

Four classes of growth factor receptor tyrosine kinases have been categorized (see Figure 14-2).[135] One class, represented by the ERBB family, was described in earlier sections. A second class includes dimeric receptors such as the insulin growth factor receptor as well as several proto-oncogenes. Members of the third class, including FMS, PDGF, FLT1, and KIT, display five immunoglobulin-like domains in the extracellular region, and the catalytic domain is interrupted by a hydrophilic

"interkinase" sequence of variable length. The fibroblast growth factor receptors represent the fourth class, which differ from the third class by having only three immunoglobulin-like domains in the extracellular region and a short kinase insert in the intracellular domain. FLT3 is a member of the third class of tyrosine kinase receptors.[136]

Particular mutations in *FLT3* aberrantly activate the FLT3 kinase and predict poor prognosis in AML. These mutations include internal tandem duplications (ITD) close to the transmembrane domain or point mutations affecting an aspartic acid residue in the kinase domain (D835 mutations). The ITD can easily be detected by PCR with primers flanking the potentially duplicated region. The size of the amplicon observed by agarose or capillary gel electrophoresis will increase in the event of an ITD.[137] D835 mutations can be detected by PCR-RFLP, where an *Eco*RV restriction site is destroyed by

Table 14.7 **Chromosomal Abnormalities Associated With Leukemias and Lymphomas**

Disease	Chromosomal Mutation
Pre-B acute lymphoblastic leukemia	t(1;19)
Acute lymphocytic leukemia	t(4;11), t(11;14), t(9;22), del(12)(p11) or (p11p13) or t(12)(p11), i(17q)
B-cell leukemia	t(2;8), t(8;14), t(8;22), t(11;14)
Acute T-lymphocytic leukemia	t(11;14), del(9)(p21 or 22)
Acute myeloid leukemia/myelodysplastic syndrome	t(11q23)-multiple partners
Acute myeloid leukemia (M2)	t(8;21), t(6;9)
Acute promyelocytic leukemia (M3)	t(15;17), 14q+
Acute myelomonocytic leukemia (M4)	t(11;21), inv(16)(p13q22)
Acute monocytic leukemia (M5)	t(9;11), del(11)(q23), t(11q23)-multiple partners
Chronic myelogenous leukemia	t(9;22), t(11;22), +8, +12, i(17q)
Acute lymphocytic leukemia	t(9;22), t(12;21), t(8;14), t(2;8), t(8;22), t(11q)
Acute nonlymphocytic leukemia	t(8;21), -Y
Chronic lymphocytic leukemia	14q+, +12, t(14;19), del(11)(q22), del(13q), del(17)(p13)
T-chronic lymphocytic leukemia	Inv(14)(q11q32) or t(14;14)(q11;q32)
Burkitt's lymphoma	t(8;14), t(2;8), t(8;22)
Diffuse large B-cell lymphoma	t(3q27), t(14;18); t(8;14)
T-cell lymphoma	t(8;14)
Follicular lymphoma	t(14;18), t(8;14)
Mantle cell lymphoma	t(11;14)
Multiple myeloma	t(14q32), 14q+
Acute nonlymphocytic leukemia	inv(3q)(q21q26),dup(3q),t(3q;3q), -5 or
Myeloproliferative/myelodysplastic disease	del(5)(q12q33), -7 or del(7)(q22), +8, +9
Myeloproliferative/myeloblastic disease	del(13)(q12 or q14), +21
Hairy cell leukemia	14q+
Waldenström's macroglobinemia	14q+
Mucosa-associated lymphoid tissue lymphoma	t(11;18), t(14;18), t(1;14)
Polycythemia vera	del(20)(q11.2q13.3)

the presence of the mutation or by real-time PCR with FRET probes.[138] In performing these assays, it is important to have adequate representation of tumor cells in the specimen to avoid false-negative results from the presence of an excess of normal cells.

Additional Chromosomal Abnormalities

Tumor suppressor genes and oncogenes are disrupted by numerous genetic events. A number of specific abnormalities are associated with particular diseases (Table 14.7). These chromosome irregularities are targets for diagnosis of the associated diseases.

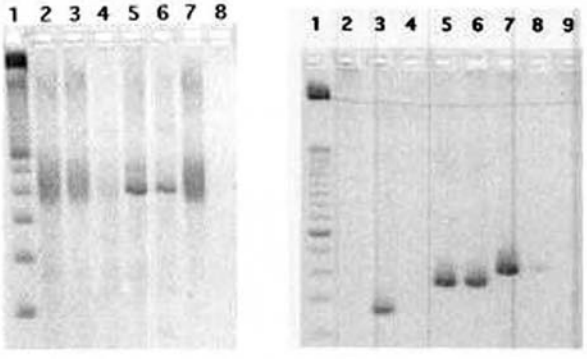
To date, many molecular oncology tests are developed by an individual library from published or original procedures rather than purchased as a set of reagents or in kit form from a commercial source. Often the final details of these procedures are determined empirically so that, in detail, a test procedure can differ from one laboratory to another. Even after the test procedure is established, trouble-shooting performed as the procedure is put to use on a routine basis may further modify the procedure. Some reactions that work well for short-term research purposes may prove to be less consistent under the demands of the clinical laboratory setting.

Biotechnology is rapidly developing standard reagent sets for the most popular tests, but these may also differ from one supplier to another. Furthermore, due to market demands, test reagent kits may be modified or discontinued. If replacement reagents are available, they may not be identical to those previously used. Ongoing tests then have to be optimized. This can be a concern where turnaround times are critical.

It then becomes the responsibility of the technologist to perform and monitor tests on a regular basis to maintain consistency and accuracy of results. The technologist who understands the biochemistry and molecular biology of these tests will be better able to respond to these problems. In addition, with the quickened evolution of the sciences, a knowledgeable technologist can better recognize significant discoveries that offer potential for test improvement.

Case Study 14 • 1

A 40-year-old woman with a history of non-Hodgkin's lymphoma reported to her physician for follow-up testing. Her CBC was normal, including a WBC count of 11,000/μL. Morphological studies on a bone marrow biopsy and a bone marrow aspirate revealed several small aggregates of mature and immature lymphocytes. Flow cytometry studies were difficult to interpret as there were too few B cells in the bone marrow aspirate specimen. No chromosomal abnormalities were detected by cytogenetics. A bone marrow aspirate tube was also sent for molecular analysis; namely, immunoglobulin heavy chain gene rearrangement and t(14;18) gene translocation analysis by PCR. The immunoglobulin heavy chain gene rearrangement results are shown in lane 4 of the gel image below:

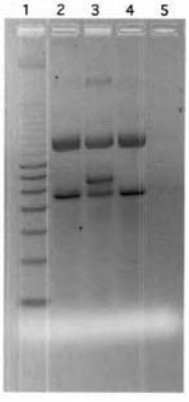


Immmunoglobulin heavy chain gene rearrangement results (left): lane 1, molecular weight marker; lanes 2 to 4, patient specimens; lane 5, positive control; lane 6, sensitivity; lane 7, negative control; lane 8, reagent blank. t(14;18) gene translocation test (right): lane 1, molecular weight marker; lanes 2 to 6, patient specimens; lane 7, positive control; lane 8, sensitivity; lane 9, negative control.

Case Study 14 • 2

A 54-year-old woman with thrombosis, a high platelet count (900,000/μL), and a decreased erythrocyte sedimentation rate was tested for polycythemia vera. Megakaryocyte clusters and pyknotic nuclear clusters were observed in a bone marrow biopsy. Overall cellularity was decreased.

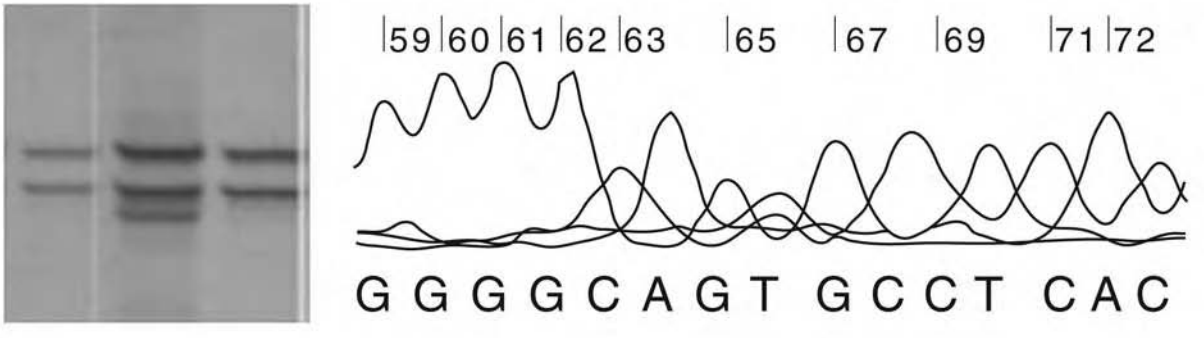
In the CBC, white blood cell and neutrophil counts were normal. Iron stores were also in normal range. A blood sample was submitted to the molecular pathology laboratory for JAK2 V617F mutation analysis. The results are shown below:



Agarose gel electrophoresis of SSP-PCR amplicons. Extension of a JAK2 V617F-specific primer produces a 270-bp band in addition to the normal 500- and 230-bp bands. Lane 1, molecular weight markers; lane 2, patient specimen; lane 3, V617F mutant control; lane 4, normal control; lane 5, reagent blank.

Case Study 14 • 3

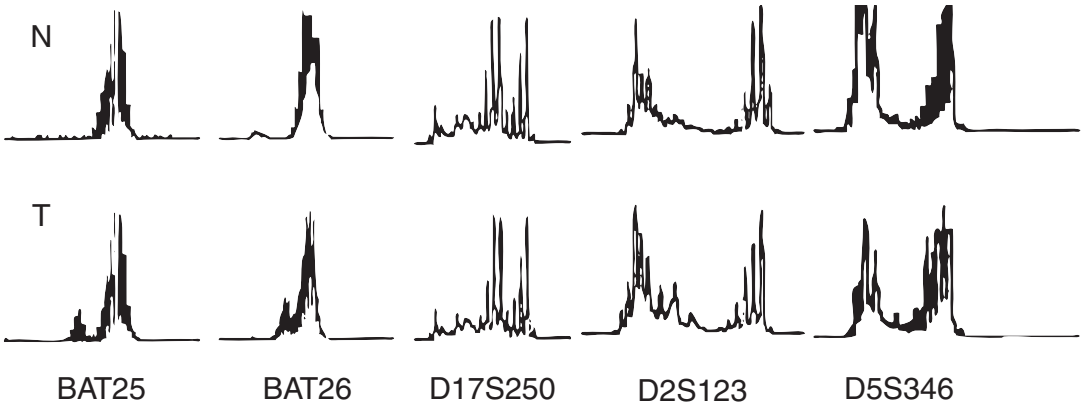
Paraffin-embedded sections were submitted to the molecular diagnostics laboratory for p53 mutation analysis. The specimen was a small tumor (intraductal carcinoma in situ) discovered in a 55-year-old woman. Lymph nodes were negative. Slides stained with hematoxylin and eosin were examined for confirmation of the location of tumor cells on the sections. These cells were dissected from the slide. DNA isolated from the microdissected tumor cells was screened by SSCP for mutations in exons 4–9 of the p53 gene. The results for exon 5 are shown below:



Polyacrylamide gel electrophoresis with silver stain detection of p53 exon 5 (left). Lane 1, normal; lane 2, patient; lane 3, normal. Direct sequencing (right) revealed a C→T (G→A) base change, resulting in a R→H amino acid change at position 175.

Case Study 14 • 4

Colon carcinoma and three polyps were resected in a right hemicolectomy of a 33-year-old man. Without a family history, the man's age and the location of the tumor warranted testing for hereditary nonpolyposis colorectal carcinoma. Histological staining was negative for MSH2 protein. Paraffin sections and a blood sample were submitted to the molecular pathology laboratory for microsatellite instability testing. DNA was isolated from tumor cells dissected from four paraffin sections and from the patient's white blood cells. Both were amplified at the five microsatellite loci recommended by the National Cancer Institute. Results are shown below:



MSI analysis of normal cells (N, top row) and tumor cells (T, bottom row) at the five NCI loci.

Case Study 14 • 5

A 23-year-old college student was sent to the student health office with a painful bruise that persisted for several weeks. He was referred to the local hospital, where the physician ordered a bone marrow biopsy and a CBC. His white blood cell count was 28,000/μL; red blood cells, 2 million/μL; hemoglobin 8, hematocrit 20, platelets, 85,000/μL. Neutrophils were 7%, lymphocytes were 5%, and blasts were 95%. The pathologist who examined the bone marrow biopsy requested flow cytometry analysis of the aspirate. The cells expressed 84% CD20, 82% CD34, 92% HLA-DR, and 80% CD10/CD19. The results of these tests indicated a diagnosis of acute lymphoblastic leukemia. A blood specimen was sent to the cytogenetics laboratory for chromosomal analysis. Twenty metaphases examined had a normal 46,XY chromosomal complement. Interphase FISH was negative for t(9;22) in 500 nucleii.

• STUDY QUESTIONS •

1. What are the two important checkpoints in the cell division cycle that are crossed when the regulation of the cell division cycle is affected?

2. An EWS-FLI-1 mutation was detected in a solid tumor by RT-PCR. Which of the following does this result support?
   a. Normal tissue
   b. Ewing's sarcoma
   c. Inherited breast cancer
   d. Microsatellite instability

3. Mutation detection, even by sequencing, is not definitive with a negative result. Why?

4. A PCR test for the bcl-2 translocation is performed on a patient with suspected follicular lymphoma. The results show a bright band at about 300 bp for this patient. How would you interpret these results?

5. Which of the following misinterpretations would result from PCR contamination?
   a. False positive for the t(15;17) translocation
   b. False negative for the t(15;17) translocation
   c. False negative for a gene rearrangement

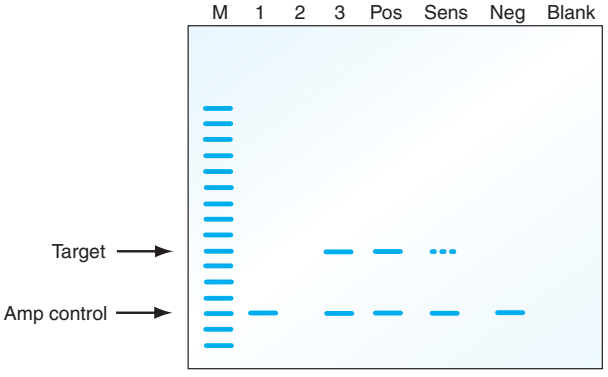
6. After amplification of the t(12;21) breakpoint by RT-PCR, the PCR products along with the proper molecular weight standard were loaded and resolved on an agarose gel. What might be the explanation for each of the following observations when the gel is exposed to ultraviolet light? (Assume that positive and amplification controls and a reagent blank control are included in the run.)
   a. The gel is blank (no bands, no molecular weight standard)
   b. Only the molecular weight standard is visible
   c. The molecular weight standard is visible; there are bands in every lane at 200 bp, even in the reagent blank lane

7. What is observed on a Southern blot for gene rearrangement in the case of a positive result?
   a. No bands
   b. Germline bands plus rearranged bands
   c. Smears
   d. Germline bands only

8. Cyclin D1 promotes passage of cells through the G1 to S checkpoint. What test detects translocation of this gene to chromosome 14?
   a. t(14;18) translocation analysis (*BCL2, IGH*)
   b. t(15;17) translocation analysis (*PML/RARA*)
   c. t(11;14) translocation analysis (*BCL1/IGH*)
   d. t(8;14) translocation analysis (*MYC/IGH*)

9. Why is the Southern blot procedure superior to the PCR procedure for detecting clonality in some cases?
   a. Southern blot requires less sample DNA than does PCR
   b. The PCR procedure cannot detect certain gene rearrangements that are detectable by Southern blot
   c. Southern blot results are easier to interpret than PCR results
   d. PCR results are not accepted by the College of American Pathologists

10. Interpret the following results from a translocation assay.



Are the samples positive, negative, or indeterminate?
Sample 1:
Sample 2:
Sample 3:

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Chapter 15 *Lela Buckingham*

DNA-Based Tissue Typing

OUTLINE

OBJECTIVES

- Describe the structure and function of the major histocompatibility (MHC) locus.
- List the human leukocyte antigens (HLAs) that are encoded by the MHC locus, and explain their role in tissue engraftment and rejection.
- Compare and contrast the levels of typing resolution that are achieved by different laboratory methods.
- Describe the laboratory methods used to identify HLAs by serology testing.
- Describe the DNA-based testing methods used for the identification of HLAs.
- Explain how combining different test methods to identify HLAs increases resolution and resolves ambiguities.
- Discuss factors, in addition to the HLAs, that affect engraftment.
- Relate the use of HLA typing for confirming disease diagnosis and predisposition.

The **major histocompatibility complex** (**MHC**) is a group of genes located on the short arm of chromosome 6. In humans, the MHC gene products are called **human leukocyte antigens** (**HLAs**). The HLAs were named for their role in rejection of transplanted organs. Kidney, heart, liver, lungs, skin, pancreas, corneas, blood, bone marrow, and hematopoietic stem cells can be transplanted from one human to another. Transplanted organs, except in the case of identical twins, are **allografts**, indicating genetic differences between the donor of the organ and the recipient. When a transplant is performed, compatibility (matching) of the HLA of the organ donor and the recipient increases the chance for a successful engraftment that will function for several years. If the donor and recipient are not HLA-matched, then the recipient's immune system (primarily mediated by T lymphocytes as well as B lymphocytes) will recognize the donor organ as non-self (foreign) and will mount an immune response against the organ, resulting in its destruction, loss of function, and rejection by the recipient.

The role of the clinical laboratory is to evaluate the HLAs of potential donors and recipients and to aid in prediction of successful engraftment and the avoidance of **graft versus host disease** (GVHD). GVHD is the reciprocal of graft rejection whereby immunocompetent cells in the donor organ recognize recipient cells as foreign and attack and destroy the recipient cells, resulting in significant morbidity and potential mortality to the recipient. The process of HLA identification utilizes methods targeting cell-associated antigens as well as serum antibodies. Most tissue typing, as HLA identification is commonly called, is performed by serological methods using antibodies to the different HLA found in the human population. Increasingly, however, DNA typing methods are being implemented for this purpose, increasing the sensitivity and specificity of the typing procedure.

The MHC Locus

The human MHC locus was discovered in the early 1950s. Investigators independently noted that blood from women who had borne children or from previously transfused persons contained antibodies that agglutinated leukocytes.[1,2] This discovery led to serological typing methods that originally identified two polymorphic gene loci, HLA-A and HLA-B, followed soon after by the identification of HLA-C and other genes. A test for typing these loci was designed from the observation that large immature mononuclear cells proliferated if lymphocytes from unrelated individuals were mixed and cultured together.[3,4] Results from this **mixed lymphocyte culture** (MLC) reaction did not always agree with the results of serological typing, however. The discrepancy was partly resolved by the discovery of additional genes comprising the HLA-D locus.[5–7]

HLAs are divided into three classes (I, II, and III), all encoded by a gene complex located on chromosome 6p (Fig. 15-1). The MHC locus includes genes other than those that code for the HLA. Cytokine genes and genes encoding tumor necrosis factor α (TNF-α) and tumor necrosis factor β (TNF-β) are located inside of the main HLA complex.[8]

Historical Highlights

The genetic contribution to transplant rejection was first proposed in 1927 when Bover observed that skin transplants between identical twins were not rejected like those from genetically distinct individuals.[70] The genes involved were first described in mice by Gorer.[71] Snell[72] used mouse cell lines to further define a genetic locus, which he called H for histocompatibility. Gorer referred to the gene products of this locus as antigen II, and the combined term H-2 was subsequently used for the MHC locus in mice.

Advanced Concepts

Despite the discovery of the MHC gene products as mediators of transplant rejection, recognition of allografts (organs from genetically different donors) is not the main function of these glycoproteins. HLAs appear on the surface of cells of the immune system, allowing cell-cell communication during immune functions. Immune reactions involve and are restricted by interactions between T lymphocytes (cells involved in cell-mediated immunity), B lymphocytes (antibody-producing cells), and the MHC molecules.[73,74]

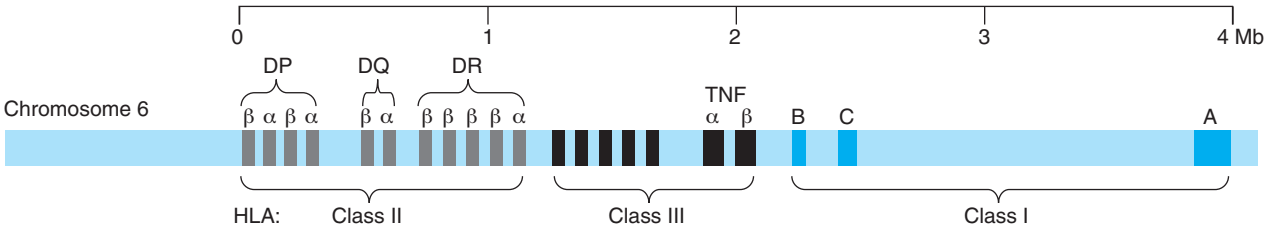


Figure 15-1 The MHC locus on chromosome 6 covers about 4 Mb of DNA, depending on the individual. Class I genes are 3–6 kb long, and class II genes are 4–11 kb in length. TNF-α and TNF-β are not part of the polymorphic HLA system.

In addition to the main MHC locus, gene regions extending beyond the HLA-DP genes toward the centromere and HLA-F toward the telomere comprise the **extended MHC locus** (xMHC). The xMHC locus covers 8 Mb and includes the hemochromatosis gene HLA-F (also called *HFE*), the farthest telomeric gene in the complex.[9,10] The most centromeric locus of the extended MHC is the tapasin region. Tapasin is required for antigenic peptide processing.[11,12] Some genes that are associated with disease conditions, such as *HFE* linked to the MHC locus, are the basis for the association of particular disease states with HLA type (see Chapter 11). The role of the immune system in other disease states, such as autoimmune diseases and susceptibility to infections, also links HLA type to disease.

The gene products of the MHC, class I, II, and III proteins, are present in different amounts on different tissues (Table 15.1). Class I and II are the strongest antigens expressed on cells. Class I molecules (designated as A, B, or C) are expressed on all nucleated cells, whereas class II molecules (designated as D) are only expressed constitutively on "professional antigen-presenting cells," such as B lymphocytes, dendritic cells, and macrophages. As illustrated in Figure 15-2, class I molecules consist of a long (heavy) chain of 346 amino acids (44 kD) associated with a smaller peptide, called **β-2 microglobulin**, which is 99 amino acids (12 kD) in size and is not encoded in the MHC. The two chains are associated with one another on the cell surface by noncovalent bonds. The class I heavy chain displays short branched-chained sugars, making this molecule a glycoprotein. The heavy chain is also a transmembrane polypeptide, anchoring the complex at the surface of the cell. Class II molecules consist of two transmembrane polypeptides, an α chain with three domains, α_1, α_2, and α_3, and a β chain, with two domains, β_1 and β_2. The two polypeptides associate, forming a groove between the α_1 and β_1 domains that will hold fragments of antigen that have been engulfed and processed by the cell (extracellular antigens). In contrast, antigens bound to class I molecules (where the peptide-binding domain is

Table 15.1 **Genes of the Major Histocompatibility Locus**

MHC Region	Gene Products	Tissue Location	Function
Class I	HLA-A, HLA-B, HLA-C	All nucleated cells	Identification and destruction of abnormal or infected cells by cytotoxic T cells
Class II	HLA-D	B lymphocytes, monocytes, macrophages, dendritic cells, activated T cells, activated endothelial cells, skin (Langerhans' cells)	Identification of foreign antigen by helper T cells
Class III	Complement C2, C4, B	Plasma proteins	Defense against extracellular pathogens
Cytokine genes	TNF-α, TNF-β	Plasma proteins	Cell growth and differentiation

Advanced Concepts

Class I and II molecules present fragments of antigens, usually about nine amino acids long, to T lymphocytes. Class I and II molecules vary from one another (are polymorphic), sometimes by a single amino acid. Due to these polymorphisms, different HLA molecules (HLA types) vary in their efficiency of binding antigen fragments, resulting in a range of immune responses to a given antigen. This distinction can affect symptoms of disease; for example, the likelihood of persons of a particular HLA type infected with HIV to develop full immunodeficiency.[68,75,76]

formed between the α_1 and α_2 domains) are generated from the processing of macromolecules synthesized within the cell (intracellular antigens).

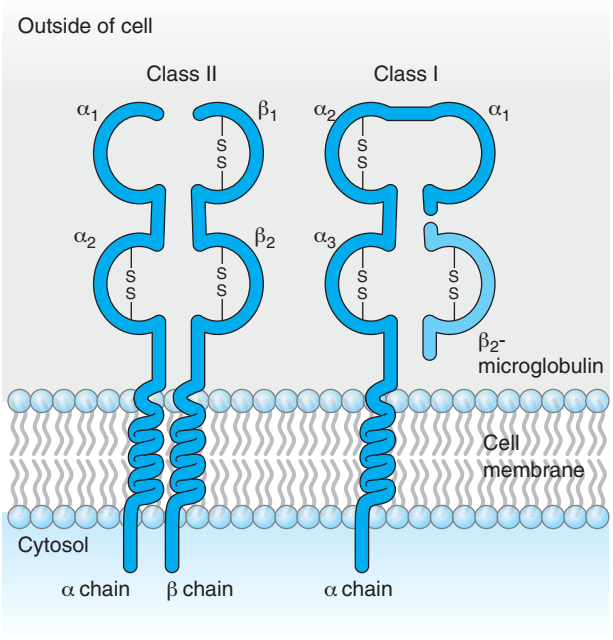


Figure 15-2 Class II (left) and class I (right) polypeptides. Class II antigens consist of two chains, α and β. Class I antigens consist of a heavy chain and a light chain associated together with a molecule of β-2 microglobulin.

HLA Polymorphisms

Genes of the MHC are the most polymorphic genes of the human genome. Polymorphisms in this locus were first defined phenotypically by acceptance or rejection of tissue or by reaction with defined antibodies (serological typing). Molecular typing methods reveal HLA polymorphisms as base changes in the DNA sequence (Fig. 15-3). The changes range from a single base pair (single nucleotide polymorphisms) to loss or gain of entire genes. A particular sequence, or version, of an HLA gene is an **allele** of that gene. The **HLA type** is the col-

(A)

```
CGG GCC GCG GTG GAC ACC TAC TGC AGA CAC AAC TAC GGG GTT GGT GAG AGC TTC ACA
CGG GCC GCG GTG GAC ACC TAT TGC AGA CAC AAC TAC GGG GCT GTG GAG AGC TTC ACA
CGG GCC GCC GTG GAC ACC TAT TGC AGA CAC AAC TAC GGG GCT GTG GNN NNN NNN NNN
```

(B)

```
CGG GCC GCG GTG GAC ACC TAC TGC AGA CAC AAC TAC GGG GTT GGT GAG AGC TTC ACA
--- --- --- --- --- --- --T --- --- --- --- --- --- -C- -TG --- --- --- ---
--- --- --C --- --- --- --T --- --- --- --- --- --- -C- -TG -** *** *** ***
```

Figure 15-3 DNA polymorphisms in the HLA-DRB1 gene. The initial sequence (DRB1*0101) is written at the top of panel A. Aligned underneath are two alleles of this region, DRB1*01022 and DRB*0103. The green bases are those that differ from DRB1*0101. N indicates unknown or unsequenced bases. Panel B shows a different way of presenting the alleles. A dash indicates identity to the consensus sequence. Only the polymorphic bases are written. The asterisk indicates unknown or unsequenced bases.

lection of alleles detected by phenotypic or genotypic typing methods.

Thus, each HLA gene can differ in sequence from one individual to another, except for identical twins. A set of particular alleles on the same chromosome is a **haplotype** (Fig. 15-4). These alleles are inherited together as a block of chromosomal sequence, unless a rare recombination within the region separates the alleles. An HLA haplotype is, therefore, the combination of polymorphic sequences or alleles in the HLA gene regions.

Polymorphisms are concentrated in exons 2 and 3 of the class I genes and in exon 2 of the class II genes. These exons code for the amino acids that interact with antigenic peptides, affecting the recognition of nonself peptides. Molecular methods target these exons in HLA-A, HLA-B, and HLA-C class I genes and mostly HLA-DRB class II genes. Other areas including introns have been investigated for potentially useful alleles.[13]

HLA Nomenclature

Polymorphisms are alterations in DNA and/or protein sequences shared by at least 2% of a defined population. Formally, alterations present at lower frequencies are called mutations or variants. Structurally, mutations and polymorphisms are the same thing, changes from a consensus amino acid or nucleotide sequence. When these changes occur in a gene, the different versions of the gene are referred to as alleles. The polymorphic nature of the MHC, therefore, means that there are multiple alleles of each HLA gene present in the human population. These alleles differ by nucleotide sequence at the DNA level (polymorphisms) and by amino acid sequence and antigenicity at the protein level. Polymorphisms arise mostly as a result of gene conversion events and rare chromosomal recombinations. Each person will have a particular group of HLA alleles inherited from his or her parents

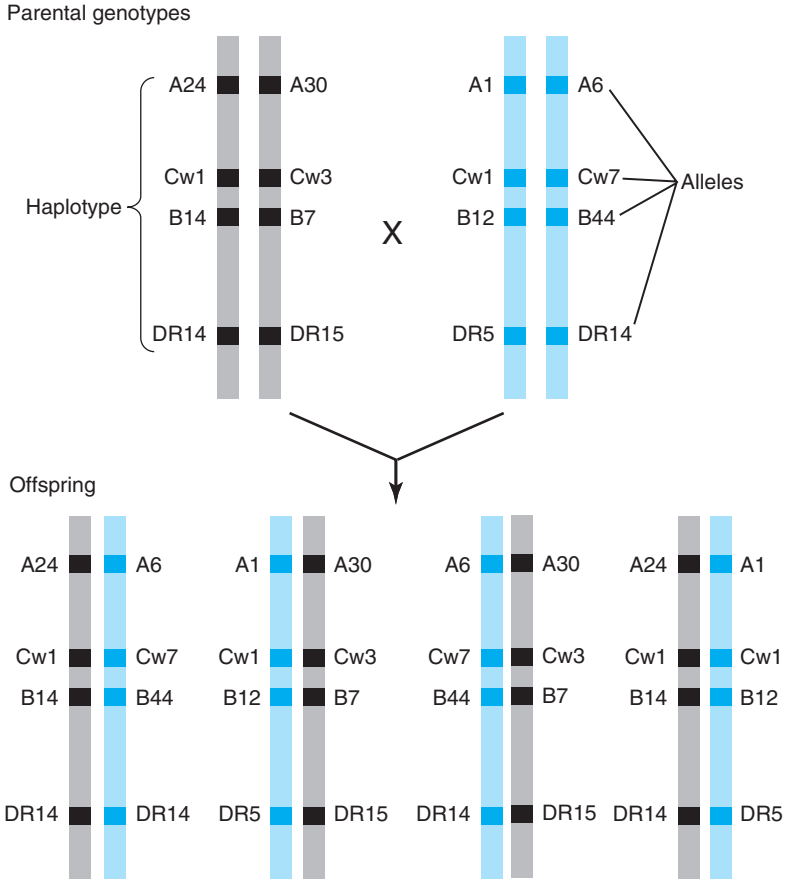


Figure 15-4 A haplotype is the combination of alleles that are inherited together. In this example, parental genotypes (top) can produce four possible genotypes in the offspring (bottom).

(see Chapter 11 on Human Identification.) The maternal and paternal HLA antigens are expressed codominantly on cells.

HLA alleles were first defined at the protein level by antibody recognition (serologically). A standard nomenclature for expressing serologically defined antigens was established by the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System. In this system, "HLA" refers to the entire gene region, and "A, B, D" refer to the particular locus; for example, HLA-A, HLA-B, or HLA-D. The HLA-D locus consists of subregions P, Q, M, O, and R, termed HLA-DP, HLA-DQ, HLA-DM, and so forth. Each of these subregions consists of genes that code for either an α or β chain polypeptide; for example, the first β polypeptide is encoded in the HLA-DRB1 gene (see Fig. 15-1). A small w is included in HLA-Cw, HLA-Bw4, and HLA-Bw6 allele nomenclature. The w denotation was originally a designation of alleles in "workshop" status or found in high prevalence in the population. The workshop designation is no longer required for the HLA-C locus; however, the w was retained for HLA-C alleles to distinguish them from the C designation used for complement genes. The w is retained with HLA-Bw4 and HLA-Bw6, which are considered "public" (high prevalence) antigens.

A list of the serologically defined alleles of the HLA genes accepted by the WHO is shown in Table 15.2. The WHO official nomenclature refers to serologically defined alleles: a number follows the gene region name; for example, HLA-B51 denotes HLA-B antigen 51 (defined by reaction to a known antibody). Number designations of new alleles of a previously-defined allele with broad specificity (parent allele) are followed by the number of the parent allele in parentheses. For example, HLA-A24(9) denotes the HLA-A antigen 24 from parent antigen 9. The derived antigens are called **split** specificities. Additional antigens have been defined by reactions between known antigens and serum antibodies (antiserum reactivity). For class I, there are 85, 188, and 42 HLA-A, B, and C alleles, respectively. For class II, there are 76, 32, and 221 HLA-DPB, DQB, and DRB alleles, respectively. These numbers will increase as new specificities are defined.

With the introduction of molecular biology techniques in the 1980s, HLA typing at the DNA level required no-

Table 15.2 **Serologically Defined HLA Specificities***

HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ	HLA-DP
A1	B5	Cw1	DR1, DR103	DQ1	DPw1
A2, A203, A210	B51(5), B5102, B5103	Cw2	DR2	DQ5(1)	DPw2
A3	B52(5)	Cw3	DR15(2)	DQ6(1)	DPw3
A9	B7, B703	Cw9(w3)	DR15(2)	DQ2	DPw4
A23(9)	B8	Cw10(w3)	DR3	DQ3	DPw5
A24 (9), A2403	B12	Cw4	DR17(3)	DQ7(3)	DPw6
A10	B44(12)	Cw5	DR18(3)	DQ8(3)	
A25(10)	B45(12)	Cw6	DR4	DQ9(3)	
A26(10)	B13	Cw7	DR5	DQ4	
A34(10)	B14	Cw8	DR11(5)		
A66(10)	B64(14)		DR12(5)		
A11	B65(14)		DR6		
A19	B15		DR13(6)		
A74(19)	B62(15)		DR14(6), DR1403, DR1404		
A68(28)	B63(15)		DR7		
A69(28)	B75(15)		DR8		
A29(19)	B76(15)		DR9		
A30(19)	B77(15)		DR10		

Continued on following page

Table 15.2 **Serologically Defined HLA Specificities*** *(continued)*

HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ	HLA-DP
A31(19)	B16		DR51		
A32(19)	B38(16)		DR52		
A33(19)	B39(16), B3901, B3902		DR53		
A36	B17				
A43	B57(17)				
A80	B58(17)				
	B18				
	B21				
	B49(12)				
	B50(12)				
	B22				
	B54(22)				
	B55(22)				
	B56(22)				
	B27, B2708				
	B35				
	B37				
	B40, B4005				
	B60(40)				
	B61(40)				
	B41				
	B42				
	B46				
	B47				
	B48				
	B53				
	B59				
	B67				
	B70				
	B71(70)				
	B72(70)				
	B73				
	B7801				
	B81				
	Bw4				
	Bw6				

*Alleles are listed in the order of the broad antigen groups, followed by split antigens for that group, with the broad antigen number in parentheses. Associated antigens, such as B40 and B4005, are listed together.

menclature for specific DNA sequences.[14–16] Many new alleles have been and are currently being defined at the DNA level.[17,18] A revised nomenclature is used for denoting alleles defined by DNA sequence. The alleles are named in sequential order as they are discovered. The gene name, such as HLA-DRB1, is followed by an asterisk, (*) the allele sequence family number, and a number for the specific allele (DNA sequence). For example, A*2503 is the third specific allele, 03, of the HLA-A*25 family of alleles.

Advanced Concepts

The WHO Nomenclature Committee has proposed addition of a third digit to the allele designation to accommodate more than 99 alleles; for example, A*2501 to A*2599 would expand to A*25999. Other ways to accommodate the growing number of reported alleles have been proposed.[19]

The letter N following the specific allele number indicates a **null allele**, or phenotypic absence of that antigen. For example, B*1307N denotes the 07 allele of the 13 allele family in the HLA-B locus at the DNA level. At the protein level, however, the encoded protein does not react with any antibody. The B*1307N null allele is due to a 15-bp deletion in the HLA-B gene. Null alleles can also result from nonsense, frameshift, splice site, or other premature stop mutations that prevent translation of the amino acids destined to bind to antibody.

Silent mutations (changes in the DNA sequence that do not change the amino acid sequence), also called **synonymous** changes, are designated by a number following the specific allele number. For example, A*030102 indicates a synonymous allele, 02, of the first specific allele, 01, from the HLA-A*03 family of alleles. A fourth number designation indicates that the synonymous change results from a polymorphism in intronic sequences beyond the coding regions (exons) of the genes. Thus, A*26070101 is the 01 subtype allele of the HLA-A*2607 allele, the second 01 indicating that the polymorphism is in an adjacent intron.

Advanced Concepts

Other descriptive designations are less frequently used, including L, S, X and ?. The letters L and S indicate poor expression of the allele at the cell surface or a soluble allele, respectively. An X notation indicates that resolution of the allele was not done, for example B*08X. A ? indicates that the resolution was not clear: B*08?.

Advanced Concepts

The National Marrow Donor Program assigns alphabetical allele codes, such as AD or RJH to allele combinations from submitted requests. Generic codes can be used with several loci and allele families. For example the combination of alleles, 1501/1501N/1511/1515/1533/1534/1557/1560, in any HLA gene is designated RDX so that B*1501/1501N/1511/1515/1533/1534/1557/1560 = B*15RDX. Allele-specific codes are used for allele combinations that include more than one serological family or that contain an N, L, or S expression character. The lists of these codes and submissions for new codes are available at www.nmdpresearch.org

Ambiguity is the recognition of two or more antigens by the same antibody, or cross-reaction, so that the exact allele cannot be called. Ambiguity is designated by "/" between the possible allele numbers or a "–" for a series of alleles in which the first and last allele are named. For example, if a typing test results in either B*0733 or B*0735, the notation is B*0733/B*0735. If a typing test indicates that the allele is either B*0733, B*0734, B*0735, or B*0736, the designation is B*0733-B*0736. Ambiguity also arises from the inability of some typing methods to assign heterozygous alleles to one or the other chromosome. A combination of methods may be used to resolve ambiguities. Family studies are also helpful in this regard.

Resolution is the level of detail to which the allele is determined. Low resolution identifies broad allele types or groups of alleles. A typing of A*26 is low resolution, which can be determined at the serological level. Typing methods that detect specific alleles in addition to identification of all serological types are at medium resolution. Typing result A*2601/A*2605/A*26010/A*2615 is medium resolution. High resolution typing procedures can discriminate between almost all specific alleles. A*2601 is high resolution determined by DNA analysis. A range of methods from serological typing to direct DNA sequence analysis affords the laboratory a choice of low, medium, or high resolution typing. Whereas low resolution is adequate for solid organ transplantation typ-

ing, bone marrow or stem cell transplants require high resolution methods.

Molecular Analysis of the MHC

There are over 1900 HLA alleles identified so far in all loci (Table 15.3). Genetic (DNA-based) typing concentrated in the HLA-A, -B, -C, and DRB1 genes has identified alleles that far outnumber the serological alleles defined for these genes. Over 400 new nucleotide sequences were defined in just 2 years, from 2002 to 2004. The WHO Nomenclature Committee devised rules for submission of new alleles for official numerical designation.[19] Allele sequences are stored in the GenBank, the European Molecular Biology Laboratory, and the DNA Data Bank of Japan databases. A list of newly reported alleles is published monthly in the journals *Tissue Antigens*, *Human Immunology,* and the *International Journal of Immunogenetics*. A comprehensive dictionary of antigen-DNA sequence allele equivalents is published periodically.[20–23]

Table 15.3 **Number of HLA Alleles Identified Serologically and by DNA Sequence***

Gene	Serology	Genetic
Class I		
HLA-A	28	207
HLA-B	29	412
HLA-C	10	100
Class II		
HLA-DRA		2
HLA-DRB1	18	271
HLA-DRB2		1
HLA-DRB3	1	30
HLA-DRB4	1	10
HLA-DRB5	1	15
HLA-DRB6		3
HLA-DRB7		2
HLA-DRB8		1
HLA-DRB9		1
HLA-DQA1		20
HLA-DQB1	9	45
HLA-DMA		4
HLA-DMB		6
HLA-DPA1		19
HLA-DPB1		93
HLA-DOA		8
HLA-DOB		8
Extended MHC		
HLA-E		6
HLA-F		1
HLA-G		14
MICA		57
MICB		18
TAP1	6	6
TAP2	4	4

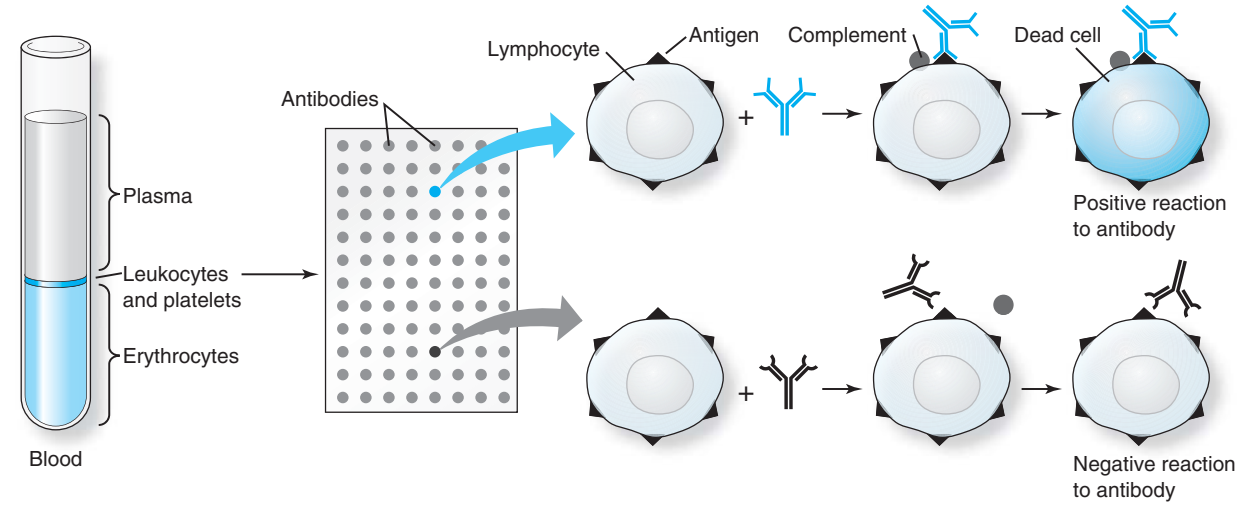
*The World Marrow Donor Association Quality Assurance and Working Group on HLA Serology to DNA Equivalents publishes a comprehensive dictionary of antigen and allele equivalents.

Identification of alleles in the laboratory serves several purposes. In addition to selection of organ donors, the extent of HLA-type matching between donor and recipient predicts the long-term survival of the donor organ in the recipient. Furthermore, because disease genes are located in and around the MHC locus, certain HLA gene alleles are linked to disease, affording another aid in diagnosis or prediction of disease phenotypes.

There are three approaches to analysis of HLA alleles in the HLA laboratory: typing, screening, and crossmatching. Typing is initial identification of the HLA alleles of a specimen through protein or DNA-based methods. Typing may be used both to define HLA haplotypes and to look for specific HLA types that are linked to disease states. Screening is detection of anti-human antibodies in serum that match known HLA alleles. Crossmatching is more specific screening of recipient sera for antibodies against antigens displayed by potential organ donors.

Serological Analysis

Traditionally, HLA typing for organ transplantation was performed serologically; that is, by antigen-antibody recognition. Although serological testing yields only low resolution typing results, there are some advantages to these methods. Serological typing is a relatively rapid method that reveals immunologically relevant epitopes. Also, serological studies can be used to resolve ambiguities or confirm null alleles detected by other methods. Serological tests include HLA phenotype determination, in which patient cells are tested with known antisera (HLA typing), and screening of patient sera for anti-HLA antibodies.

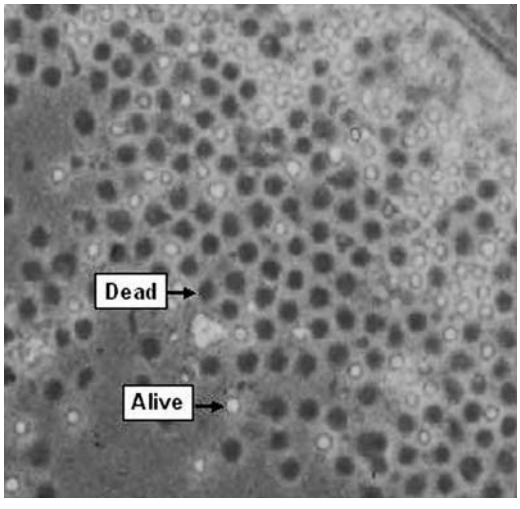


■ Figure 15-5 Crossmatching to known antibodies is performed on lymphocytes (buffy coat, left) in a 96 well plate format where each well contains different known antibodies. If the antibody matches the cellular antigen (positive reaction, top), complement-dependent cytotoxicity will occur, and the dead cell will take up stain (green). If the antibody does not match the cellular antigen, there is no cytotoxicity.

HLA Typing

Lymphocytes are HLA-typed using the **complement-dependent cytotoxicity** test (CDC; Fig. 15-5).[24] In this procedure, multiple alleles are determined using a panel of antibodies against known HLA types. These antibodies are prepared from cell lines or from donors or known HLA types. Plates preloaded with antibodies (**typing trays**) are commercially available. Alternatively, some laboratories construct their own antibody panels. The collection of antibodies can be modified to represent ethnic populations or antigens of high prevalence in particular geographical areas. As the antibody preparations are used repeatedly, the antigen binding characteristics of the various antibodies are recognized and recorded. Experienced technologists have detailed documentation of antibody panels, including which antibodies bind antigen well and which antibodies bind less strongly.

To begin the typing procedure, different antibodies are placed in each well of the typing tray. Donor or recipient lymphocytes to be typed are distributed to the wells. Cross reactivity is assessed by the uptake of trypan blue or eosin red dye in cells that have been permeabilized due to reaction with the antibody and with complement that is activated by the antigen-antibody complexes (Fig. 15-6). Cytotoxicity is scored by the estimated percentage of cells in a well that have taken up the dye. The American Society for Histocompatibility and Immunogenetics (ASHI) has developed guidelines for numerical description of the observed cytotoxicity (Table 15.4). High cytotoxicity (reading >6) in a well of the plate indicates that the cells being tested have cell surface antigens matching the known antibody in that well. As reading is somewhat



■ Figure 15-6 Cells stained for cytotoxicity. Dead cells take up dye, and live cells remain transparent. (Photo courtesy of Dr. Andres Jaramillo, Rush University Medical Center.)

Table 15.4 **Expression of CDC**

% Dead or lysed (dyed)	Interpretation	Score
0–10	Negative	1
11–20	Doubtful negative	2
21–50	Weak positive	4
51–80	Positive	6
81–100	Strong positive	8
	Unreadable	0

subjective, it is recommended that trays be read by at least two technologists independently. An example of partial results from a CDC test is shown in Table 15.5. The results indicate that the tested cells are HLA-type A28, B44.

Each HLA antigen has multiple epitopes, some unique to that HLA antigen and some **cross-reactive epitope groups** (CREG) shared by other HLA antigens. The CDC test can be performed with **private** antibodies, those that bind to one specific HLA type or antibodies to broad CREG (or "**public**" antigens). Analysis of antibodies with shared specificities aids in narrowing the specificity, as illustrated in Table 15.5. As shown, all antibodies with A28- or B44-specificity generated cytotoxicity, supporting the determination of an A28, B44 haplotype.

CREG matching or residue matching (determined from the amino acid sequences of the antigens) is considered for kidney transplant screening in order to define the spectrum of HLA-specific antibodies more precisely.

Table 15.5 **Example of Results From a CDC Assay**

Antibody	Score*
A2, A28, B7	8
A2, A28	6
A10	1
A10, A11	1
B7, B42	8
B7, B27	8
B7, B55	8
B44, B45, B21	6
B44, B45	8
B44	8
B45	1

*Scores are 1–8, depending on the percentage of dyed cells observed.

Some epitopes are more important than others with respect to organ rejection. Therefore, certain mismatches are allowable if the critical epitopes match.

Screening

Successful organ transplant depends on minimal reaction of the recipient immune system to the antigens of the donor organ. Normal sera do not have antibodies against human antigens, termed **anti-human antibodies** or **alloantibodies**. Persons who have had a previous organ transplant, blood transfusions, or pregnancies, however, will have anti-human antibodies (termed **humoral sensitization**) that may react against a new donor organ. The chance of a successful transplant is improved by defining the specificity of the alloantibodies and selecting a suitable organ that does not have HLA antigens corresponding to the antibodies in the patient's sera.[25] Humoral sensitization and the identity of alloantibodies present in the recipient serum are determined in a modified version of the CDC assay using the patient's serum as the source of antibodies and reference lymphocytes of known HLA types prevalent in the general population. The reference lymphocytes are defined by their recognition of **panel reactive antibodies** (PRA). This test against PRA reveals the percentage of the general population with whom the patient will cross-react.

The percentage of the panel of lymphocytes killed by the sera is referred to as %PRA. Patients with %PRA activity of more than 50% are considered to be highly sensitized; finding cross-match–negative donors is more difficult in these cases.[26]

Screening of sera with microparticles (beads) is performed in laboratories with flow cytometry capability (Fig. 15-7). For this method, microparticles are attached to pools of antigens derived from cell lines of defined HLA types. The microparticles are exposed to test serum, and those beads carrying antigens that are recognized by antibodies present in the test serum will bind to those antibodies. After removal of unbound antibodies, a fluorescently labeled secondary reporter antibody is applied, and the antibody-bound beads are detected by flow cytometry. The advantages of this method over the CDC test are that the reaction is performed in a single tube and there is less subjectivity in the interpretation of results. Because this test uses pooled antigens, however, it can detect prevalence of anti-human antibodies in the test serum but not identify which specific antibodies are pres-

ent. A negative result does preclude further alloantibody assessment. A variation of this method developed by Luminex Corp. and MiraiBio utilizes beads with their own internal fluorescence. By conjugating known antigens to beads of different internal fluorescence, the positively reacting antibodies can be identified while still performing the test in the same tube.

Crossmatching

The CDC test is also used for crossmatching potential organ donors and recipients. For crossmatching, recipient serum is the source of antibodies tested against donor lymphocytes (Fig. 15-8). If the recipient serum kills the donor lymphocytes, it is a positive crossmatch and contraindication for using the crossmatched donor.

Other methods used for crossmatching include variations on the lymphocytotoxicity assay and nonlymphocytotoxic methods that utilize flow cytometry, such as the bead arrays just described. Alternative methods also include enzyme-linked immunosorbent assay (ELISA) using solubilized HLA antigens. ELISA can be used to monitor the change in antibody production over time or humoral sensitization developing after the transplant.

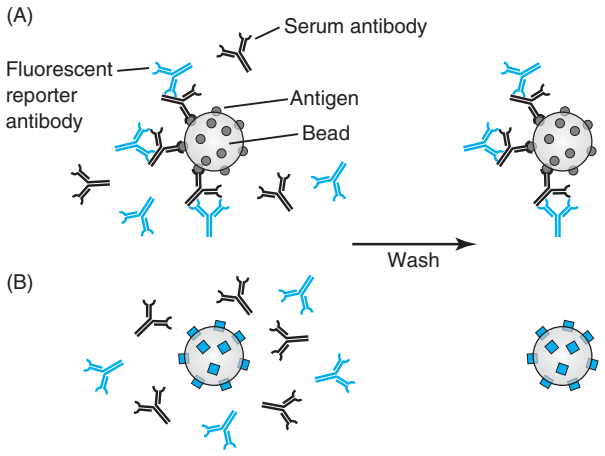


Figure 15-7 Detection of serum antibodies using bead arrays. In this illustration, separate preparations of beads are conjugated to two different known antigens. The patient serum tested contains an antibody to the antigen on the beads in (A) but not the antigen on the beads in (B). A secondary antibody targeting the bound serum antibody generates a fluorescent signal detected by flow cytometry. If a matching antibody is not present in the test serum as in (B), no antibody will be bound.

Advanced Concepts

More detailed crossmatch information is achieved by separate analysis of B and T donor lymphocytes. Unactivated T cells display class I antigens, and B cells display both class I and class II antigens. Therefore, if B cells cross react with the serum antibodies and T cells do not, the serum antibodies are likely against class II antigens.

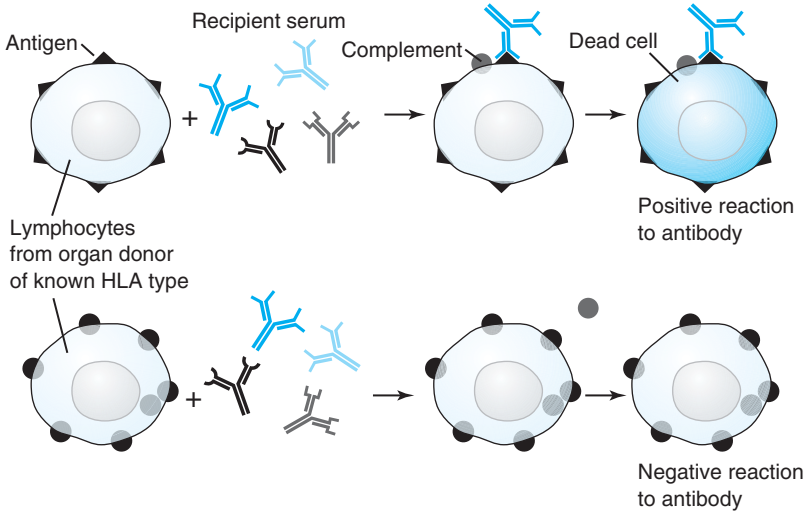


Figure 15-8 Crossmatch by CDC. In this assay, the recipient serum is the source of antibodies to type lymphocytes from potential organ donors. The antibodies in the recipient serum can be identified if the HLA type of the lymphocytes is known.

Mixed Leukocyte Culture

T lymphocytes are primarily responsible for cell-mediated organ rejection. The mixed leukocyte culture (MLC) is an in vitro method used to determine T-cell cross reactivity between donor and recipient. The MLC assay measures growth of lymphocytes activated by cross reactivity as an indication of donor/recipient incompatibility. MLC can also test for cell-mediated cytotoxicity and cytokine production, by either the donor or recipient lymphocytes, so it can be used to predict GVHD as well as recipient-mediated transplant rejection. For this test, cells must be incubated together for several days. Cell activation and growth are assessed by uptake of ^{3}H-thymine. Even though the MLC is more likely to detect HLA mismatches than serology techniques, the time and technical demands of the MLC precludes its routine use for pretransplant histocompatibility testing in the clinical laboratory.

Protein Gel Electrophoresis

The protein products of the HLA genes can be distinguished by mobility differences in one-dimensional gel isoelectric focusing or two-dimensional gel electrophoresis methods.[27,28] These typing methods have also been applied to forensic identification.[29] Protein methods are limited by the demands of the methodology and the ability to distinguish only those proteins that have different net charges. With the advent of proteomics, the use of protein markers to evaluate tissue and organ transplantation may return.[30]

DNA-Based Typing

Many people are waiting for transplant organs. Typing, screening, and crossmatch analysis are critical for selection of potential donors and successful engraftment. Methods differ in sensitivity. The choice and design of the method used, therefore, will affect the ability to predict rejection risk.

Limitations of serological and protein-based methods have led to the development of more refined molecular DNA typing with higher powers of resolution, especially for bone marrow transplantation typing. One of the first DNA methods for molecular typing was restriction fragment length polymorphism (RFLP) analysis by Southern blot. This method was used to identify HLA class II alleles.[31,32] (See Chapter 6 for a description of the Southern blot technique.) Studies showed that kidneys matched by RFLP typing survived longer than those matched by serological typing.[33,34] Just as with other applications of molecular testing, the development of amplification and direct sequencing methods greatly advanced the analysis of HLA polymorphisms at the DNA sequence level.

DNA typing focuses on the most polymorphic loci in the MHC, HLA-B, and HLA-DRB. HLA-A, HLA-B, HLA-C, and HLA-DRB are all considered important for successful transplantation outcomes.[35] For this reason, the number of alleles in these particular loci has risen significantly compared with the number of serologically defined polymorphisms.

Whole blood patient specimens collected in ethylene diamine tetra-acetic acid anticoagulant are used for DNA-based typing. Cell lines of known HLA type are used for reference samples. Standards and quality assurance for DNA-based assays have been established by ASHI (www.ashi-hla.org). DNA isolation (see Chapter 4) can be performed from white blood cell preparations (buffy coat) or from isolated nuclei treated with proteinase K.

Sequence-Specific Oligonucleotide Probe Hybridization

Hybridization of a labeled probe to immobilized amplicons of the HLA genes (dot blot) was one of the first methods that utilized polymerase chain reaction (PCR)–amplified DNA for HLA typing.[36] For this procedure (Fig. 15-9), the HLA region under investigation is amplified by PCR using primers flanking the polymorphic sequences. Because the majority of polymorphic sequences are located in exon 2 of the class II genes and exons 2 and 3 of the class I genes, primers are designed to target these regions.

An assay using 30 probes requires approximately 70 μl of PCR product.[37] The amplicons are denatured by addition of NaOH and spotted onto a membrane in 1–2–μl volumes. Spotting is done manually with a multichannel pipet or by a vacuum manifold with a 96 well plate format. The spotted DNA is dried, then permanently attached to the membrane by ultraviolet cross-linking (exposure to ultraviolet light) or baking. Separate membranes are produced for each probe to be used. Every membrane should include reference amplicons complementary (positive control) and noncomplementary (negative control) to all probes in the assay. Spotting consistency may be checked using a consensus probe that will hybridize to all specimens on a membrane.

The probes used in this assay are short (19–20 bases) single-stranded DNA chains (oligonucleotides) designed

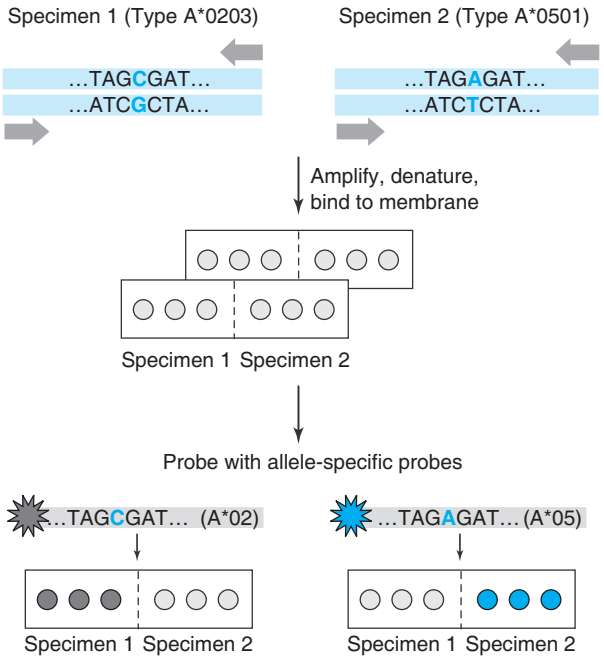


Figure 15-9 The principle of the SSOP assay is shown. An HLA gene region is amplified from specimen DNA using generic primers (top). The amplicons are immobilized on a membrane and probed with labeled sequences complementary to specific alleles. Signal from the bound probe will indicate the allele of the immobilized DNA.

to hybridize to specific HLA alleles. The oligonucleotides are labeled; that is, covalently attached to biotin or digoxygenin. (See Chapter 6 for a description of nonradioactive signal detection.) Probe sequences are based on sequence alignments of HLA polymorphic regions. The probe sequences are aligned so that the polymorphic nucleotide is in the middle of the probe sequence. Hybridization conditions depend on the optimal binding of the probe matching a test sequence in comparison with another sequence, differing from (not complementary to) the probe by at least one base. Spots of immobilized specimens bound to specific probes give a positive colorimetric or chemiluminescent signal. Panels of probes define specific alleles according to which probe binds to the immobilized amplified DNA under investigation. The number of probes used depends on the design of the assay. For example, an intermediate resolution assay of the HLA-DRB locus might take 30–60 probes. Studies have achieved high resolution identification of the majority of HLA-A, B, and C alleles using 67 HLA-A, 99 HLA-B, and 57 HLA-C probes and intermediate resolution with 39 HLA-A and 59 HLA-B alleles.[38]

Sequence-specific oligonucleotide probe (SSOP) may also be performed in a reverse dot blot configuration in which the allele-specific probes are immobilized on the membrane (Fig. 15-10). In this method, the specimen DNA is labeled by PCR amplification using primers covalently attached to biotin or digoxygenin at the 5′ end. In contrast to the SSOP described above where amplicons from each specimen are spotted on multiple membranes, each specimen is tested for multiple alleles on a single membrane. Therefore, instead of having a separate membrane of multiple specimens for each probe, a separate membrane of multiple probes is required for each specimen. Membranes with immobilized probes, such as Dynal RELI, SSO HLA-Cw, and DRB typing kits are commercially available.

A bead array system has been applied to the reverse dot blot strategy where fluorescently distinct beads carry the oligonucleotide probes (LABType SSO, One Lambda, Inc.). This system is commercially available for typing of HLA-A, B, C class I, and HLA-DRB1, DRB3, DRB4, DRB5, and DQB1 antigens.

SSOP is considered low to intermediate resolution, depending on the number and types of probes used in the assay. Probe panels are listed by the 13th International

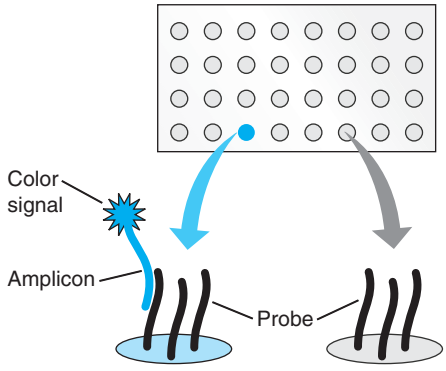


Figure 15-10 In reverse dot blot SSOP, the probe is immobilized on the membrane. Patient DNA is amplified using primers covalently bound to biotin or digoxygenin at the 5′ end. The amplicons are then hybridized to panels of probes immobilized on a membrane (top). If the sequence of the amplicon matches and hybridizes to that of the probe, a secondary reaction with enzyme-conjugated avidin or antidigoxygenin will produce a color or light signal when exposed to substrate. If the sequence of the amplicon differs from that of the probe, no signal is generated (bottom right).

Histocompatibility Workshop at www.ikwg.org and the National Marrow Donor Program (NMDP) at www.nmdp research.org. As some probes have multiple specificities, hybridization panels can be complex. Computer programs may be used for accurate interpretation of SSOP results.

Sequence-Specific PCR

A faster method of sequence-based typing is the use of sequence-specific primers that will amplify only specific alleles (Fig. 15-11). As described in Chapter 7, the 3′ end of a PCR primer must be complementary to the template for recognition by DNA polymerase. By designing primers that end on the polymorphic sequences, successful generation of a PCR product will occur only if the test sequence has the polymorphic allele complementary to the primer. Detection of the PCR product is used to indicate specific alleles. Sequence-specific PCR (SSP-PCR) is faster and easier than SSOP in that no probes or labeling steps are required and the results of SSP-PCR are determined directly by agarose gel electrophoresis.

For SSP-PCR, isolated DNA is amplified using sets of primers designed to specifically amplify a panel of alleles. Reactions are set up in a 96 well plate format, with different allele- or sequence-specific primer sets in each well. Each PCR reaction mix contains sequence-specific primers and amplification control primers in a multiplex format. The amplification control primers should yield a product for every specimen (except the negative control). The sequence-specific primers should only yield a product if the specimen has the allele complementary to (matching) the allele-specific primer sequence. The amplification primers are designed to yield a PCR product of a size distinct from the product of the allele-specific primers. The two amplicons can then be resolved by agarose gel electrophoresis. An illustration of the results expected from SSP-PCR is shown in Figure 15-12. Specimens will yield two PCR products (amplification control and allele-specific product) only from those wells containing primers matching the specimen HLA allele. Wells containing primers that do not match the patient's HLA allele will have a band only from the amplification control.

PCR plates preloaded with reaction mixes (typing trays) containing primers specific for class I and class II DRB and DRQ genes are commercially available (MicroSSP, One Lambda, Inc., and Pel Freez SSP UniTray). To use these products, specimen DNA is introduced into the individual wells, and the plates are placed in the thermal cycler. Plate maps of allele-specific primers are provided for interpretation of the HLA-type. SSP-PCR has become a commonly-used method for testing potential donors before transplant.

Sequence-Based Typing

The most definitive way to analyze DNA at the nucleotide sequence level is by direct DNA sequencing. This is true for any DNA test, no less for discovery and identification of HLA types. (See Chapter 10 for a description of DNA sequencing methodology.) Sequence-based typing (SBT) involves amplification of polymorphic regions; for example, exons 2 and 3 of the HLA-B gene (Fig. 15-13). The amplicons are then purified and added to a sequencing reaction mix (Fig. 15-14). Following gel or capillary gel electrophoresis, the fragment patterns or electropherograms are examined for specific polymorphisms. Because

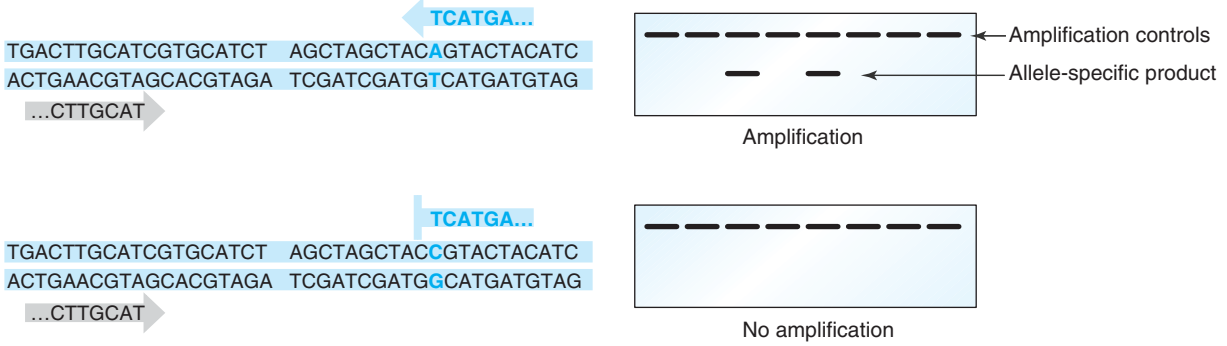


Figure 15-11 The sequence-specific primer ending in AGTACT will be extended only from a template carrying the polymorphism shown.

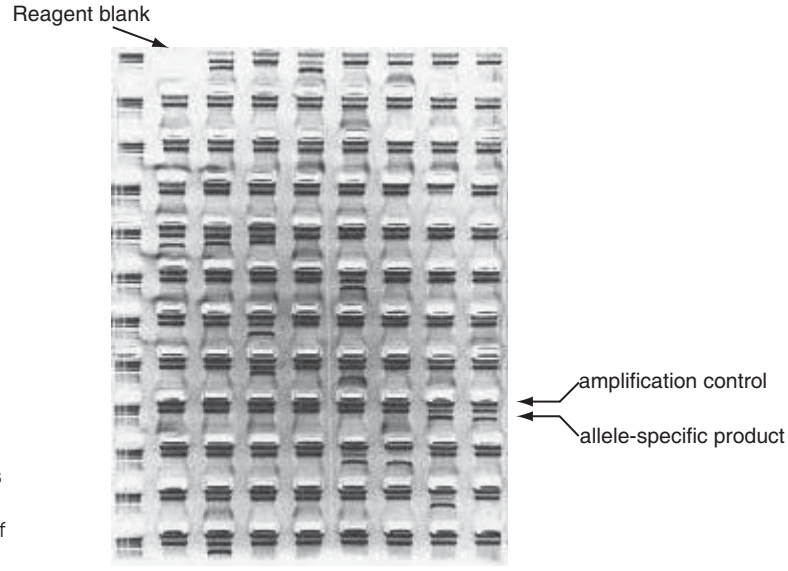


Figure 15-12 Results of an SSP-PCR of 95 primer sets detected by agarose gel electrophoresis. An amplification control is included with each reaction to avoid false-negative results due to amplification failure. Contamination is monitored by a reagent blank. (Photo courtesy of Christin Braun, Rush University Medical Center.)

HLA haplotypes are almost always heterozygous, the sequencing results often yield a heterozygous pattern at the site of the polymorphism (Fig. 15-15). Commercial systems including PCR and sequencing reaction mixes as well as software programs for interpretation of results are available.

Secondary structure and other artifacts may alter the mobility of some fragments and complicate interpretation. Furthermore, a number of events can occur that will compromise sequence quality (see Chapter 10). Manufacturers of sequencing reagents supply ways to correct most of these problems. As with any sequencing assay, it is useful to sequence both strands of the test DNA.

Other DNA-Based Methods

Almost any method that can determine DNA sequence or detect specific sequences or sequence differences can be applied to DNA-based HLA typing (see Chapter 9). There

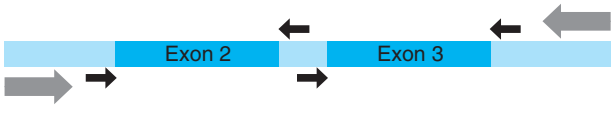


Figure 15-13 Example of primer placement for amplification and sequence analysis. PCR primers (outer large arrows) are used to amplify the region of interest. The PCR product is then sequenced using four different primers (inner small arrows) in separate sequencing reactions.

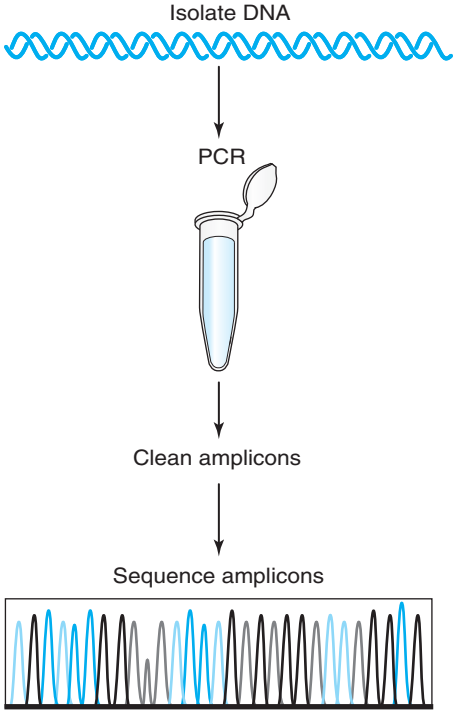
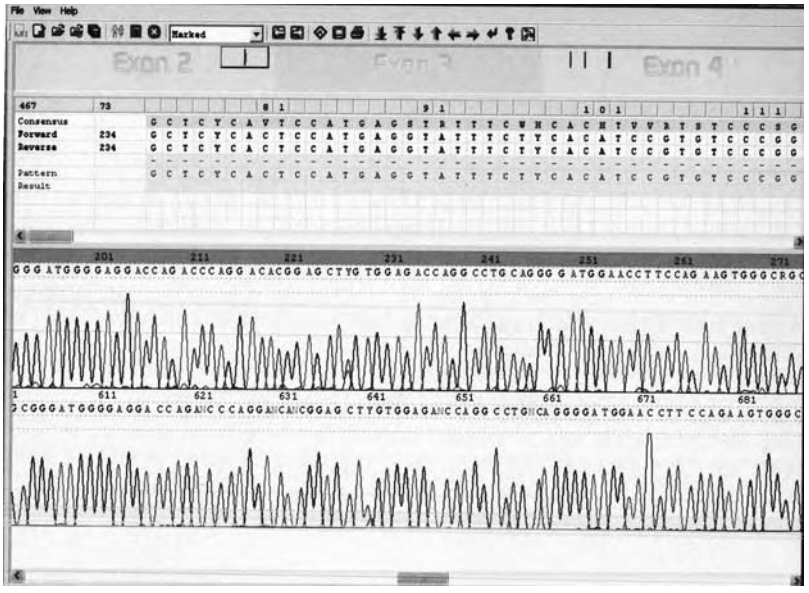


Figure 15-14 For sequenced-based typing, HLA regions from patient DNA are amplified by PCR. The PCR products are then purified from unused PCR reaction components by alcohol precipitation or column or gel purification methods. The amplicons are then sequenced to detect polymorphisms.



■ Figure 15-15 A sequence-based typing result for the HLA-A gene. Software is designed to analyze the sequence of patient DNA, compare it with the consensus sequence, and identify the specific alleles by sequence polymorphisms. (Sequence courtesy of Christin Braun, Rush University Medical Center.)

are several variations on SSOP, SSP, and SBT, such as nested PCR-SSP[39] proposed for HLA typing. Another example is allele-specific nested PCR-SSP, which has been applied to subtyping of highly polymorphic alleles.[40,41]

Heteroduplex (HD) analysis has been used for assessment of compatible bone marrow donors at the HLA-DR and HLA-DP loci.[42] Reference strand conformation polymorphism is a variation on the standard HD analysis; sample amplicons are mixed with fluorescently labeled reference DNA of known allele sequence before denaturation and renaturation to form heteroduplexes.[43] The homo- and heteroduplexes formed between the specimen amplicons and the reference strand are then resolved by capillary gel electrophoresis.

Single strand conformation polymorphism (SSCP) has been applied to HLA-A, DR, DQ, and DP typing and subtyping.[44,45] SSCP has been coupled with allele-specific PCR.[46] High performance liquid chromatography (HPLC) has been proposed for HLA typing as well. Conformation analyses, such as HD, SSCP, and HPLC, however, are limited by the complexity of the raw data and the strict demands on reactions and electrophoresis conditions; they are not routinely used in the clinical laboratory.

Other DNA-based typing approaches include array technology[47] and pyrosequencing.[48] No one method is without disadvantages with respect to technical demands, cost, or time consumption. To date, SSP, SSOP, and SBT are the DNA-based methods used in most clinical laboratories.[37,49]

Combining Typing Results

At the DNA level, HLA polymorphisms differ from one another by as small as a single nucleotide base. Serological typing does not always distinguish subtle genetic differences between types. Serological typing also requires the proper specimen. A specimen consisting of mostly T cells, for example, from a patient treated with chemotherapy, will not provide B cells (which carry class II antigens) for testing of class II haplotypes. In contrast, DNA-based typing is not limited by specimen type as all cells have the same HLA haplotype at the DNA level, regardless of whether the cell type expresses the antigens. Furthermore, synonymous DNA changes and polymorphisms outside of the protein coding regions may not alter antigenicity at the protein level.

For DNA-based methods, the design of molecular methods (primer and probe selection) determines their level of resolution. SSOP and SSP methods require specific primers and/or probes for each particular HLA type. Only those HLA-types included in a given probe or primer set, therefore, will be identified. Sequence-based typing will only identify alleles included in the amplified regions that are sequenced.

Results from serological and DNA-based methods can be combined to improve resolution and further define HLA types. Sequential use of SSP-PCR and PCR-RFLP or SSOP-PCR and SSP-PCR increases the typing resolution of DNA-based tests.[37,50] Serological testing can be used to clarify or confirm the phenotype of alleles detectable by DNA-based methods. The resolution of results from various methods, therefore, reflects a range of resolution levels (Table 15.6). The choice of method will depend on the demand for high or low resolution typing.

HLA Test Discrepancies

HLA typing may produce discrepant results, especially if different methods are used to assess the same specimen. The most common discrepancies are those between serology and molecular testing results.[51] DNA sequence changes do not always affect protein epitopes. A serology type may represent several alleles at the DNA level. Also, a serology type may look homozygous (match to only one antibody) where the DNA alleles are heterozygous, the second allele not recognized by serology. For example, a serology type of A2 is determined to be A*02, A*74 at the DNA level.

Discrepancies also arise when HLA types assigned to parent alleles based on DNA sequence homology differ from serology results that detect the same allele as a new antigen. For example, a DNA allele of B*4005 is detected as a new antigen by serology and named B50. Similarly, split alleles (subtypes of serologically defined antigens) can differ between DNA and serology typing due to the cross reactivity of antibodies used to define the HLA antigens.

The identification of new alleles can result in discrepant retyping results based on the recognition of new alleles that were not defined at the time of an initial typing. These discrepancies may be difficult to resolve, especially if the original typing data are not accessible.

Table 15.6 **Resolution of HLA Typing Methods**

Low Resolution	Intermediate Resolution	High Resolution
CDC (serology)	PCR-SSP	PCR-SSP
PCR-SSP	PCR-SSOP	PCR-SSOP
PCR-SSOP	PCR-RFLP	SSP-PCR + PCR-RFLP
		SSOP-PCR + SSP-PCR
		SBT

Coordination of HLA Test Methods

Choice of the appropriate method and resolution of HLA testing are influenced by the type of transplant. For solid organ transplants, antibody screening and crossmatching of recipient serum against donor antigens are routinely performed, although not always before transplantation. Pretransplant HLA typing is often determined for kidney and pancreas transplants, as the extent of HLA matching is directly proportional to time of survival of the donor organ.[52,53] Given the circumstances under which heart, lung, and liver transplants are performed, testing is frequently performed after the transplant. HLA typing for solid organs is usually at the low resolution serology level, although for heart and lung transplants, as with kidney transplants, studies have shown that matching HLA types are beneficial for organ survival.[54,55] For stem cell and bone marrow transplants, typing to high resolution (specific alleles) is preferred in order to decrease the risk of rejection and to avoid GVHD.[56,57]

Additional Recognition Factors

Minor Histocompatibility Antigens

Any donor protein that can be recognized as nonself by the recipient immune system can potentially affect engraftment. Proteins outside the MHC that influence graft failure are called **minor histocompatibility antigens** (mHag). These antigens were the suspected cause of GVHD and graft rejection in MHC-identical transplants.[58,59] The H-Y antigen was the first characterized mHag.[60] Investigations using molecular methods have led to the characterization of additional mHags, including HA-1, CD31, HPA-1, HPA-2, HPA-3, and HPA-5.[61] Evaluation of mHags in stem cell transplants can be carried out by molecular methods such as SSP-PCR.[62]

Nonconventional MHC Antigens

Located within the MHC locus are the MHC class I related *MICA* and *MICB* genes. Three pseudogene fragments, *MICC, MICD*, and *MICE* are also found within

the class I region. The products of the *MICA* and *MICB* genes along with those of the retinoic acid early transcript (RAET) gene cluster located on the long arm of chromosome 6 (6p21.3) bind to the receptor NKG2D on natural killer (NK) cells (killer cell lectin-like receptor, subfamily K, number 1 or KLRK1). These gene products participate in immune reactions against abnormal cells such as tumor cells through control of NK cells and cytotoxic T lymphocytes (CTL) expressing the γδ T-cell receptor. Virus- or bacteria-infected cells may also be recognized and eliminated in part by this system.

The *MICA* and *MICB* genes are highly polymorphic. Approximately 60 *MICA* and 25 *MICB* alleles have been reported. In contrast to the MHC class I alleles, polymorphisms in the *MIC* genes are distributed throughout the coding regions, with no hypervariable regions. Anti-MIC antibodies have been detected after organ transplantation, similar to anti-HLA alloantibodies, supporting a role for these gene products in organ rejection.[63]

Killer Cell Immunoglobulin-like Receptors

NK cells and some memory T cells express killer cell immunoglobulin-like proteins (KIR). The effect of these proteins was first observed as "hybrid resistance" in mice.[64] In these experiments, mice with compromised immune systems were still capable of rejecting grafts from unrelated mice. That is, graft rejection still occurred, even in the absence of a functional immune system. The KIR proteins have been proposed as one source of nonself recognition outside of the MHC. The KIR proteins interact with HLA antigens, specifically recognizing HLA-A, HLA-B, and HLA-C (class I) molecules. KIR proteins are also expressed on myelomonocytic lineage cells (leukocyte immunoglobulin-like receptor) and other leukocytes (leukocyte-associated immunoglobulin-like receptor). A cluster of genes coding for these receptor proteins has been found on chromosome 19q13.4, the **leukocyte receptor cluster** (Fig. 15-16).

Recipient KIR may participate in graft rejection and donor KIR in GVHD.[65] Specific interactions between KIR and HLA genes are listed in Table 15.7. Just as with mHags, assessment of polymorphisms in KIR may be added to donor selection criteria in stem cell and bone marrow transplant, especially with unrelated donors.[66, 67] In contrast to HLA typing, testing for KIR is aimed at finding donors and recipients who do *not* match. A KIR SSO commercial system using bead array technology is available for typing of 16 KIR genes and variants (One Lambda, Inc.).

MHC Disease Association

Genetic diseases caused by single gene disorders obey mendelian laws. Their phenotypes are either dominant or recessive and are inherited in a predictable manner illustrated in pedigrees (see Chapter 13). Most diseases, however, are not caused by a single genetic lesion and therefore have complex segregation patterns. Multiple genes, epigenetics, and environmental factors combine to bring about these disease states. For diseases such as diabetes, high blood pressure, and certain cancers, genetic analysis yields results in terms of predisposition, probability, and risk of disease.

Autoimmune diseases, which affect 4% of the population, fall in this category. At least one of the genetic factors involved in autoimmunity is linked to the MHC, as autoimmune diseases have MHC associations. Rheumatoid arthritis, multiple sclerosis, diabetes mellitus type 1, and systemic lupus erythematosus are associated with particular HLA haplotypes. Determination of a disease-

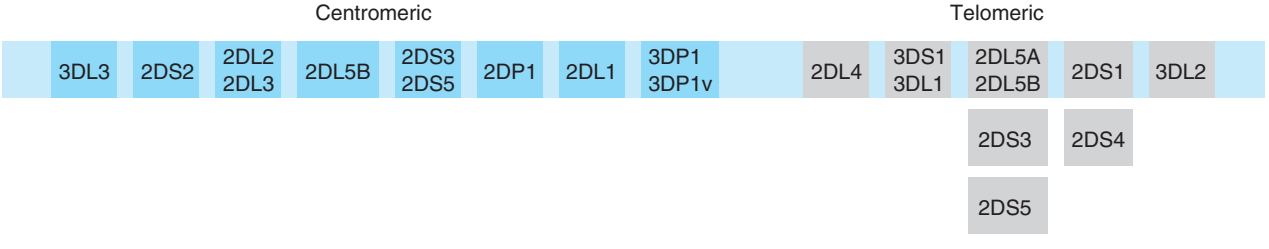


Figure 15-16 The KIR gene cluster includes a centromeric and a telomeric fragment. Gene content varies from one person to another. A KIR haplotype can contain from 8 to 14 genes in different combinations and gene orders. For example a haplotype may have a 2DL2 or 2DL3 gene between 2DS2 and 2DL5B genes, or the order of 2DS1 and 2DS4 can be 2DS1–2DS4 or 2DS4–2DS1.

Table 15.7 **KIR and HLA Gene Interactions**

KIR	HLA Specificities
2DL1, 2DS1, (2DS4)	Cw*02, *0307, *0310, *0315, *0304, *0305, *0306, *0707, *0709, *1204, *1205, *15 (except *1507), *1602, *17, *18
2DL2, 2DL3, (2DS2)	Cw*01, *03, (except *0307, *0310, *0315, *07 (except *0707, *0709), *0708, *12 (except *1204, *1205), *13, *14, *1507, *16 (except *1602)
2DL4	G
2DL5	Unknown
3DL1	Bw4
3DL2	A3, A11
3DL3	Unknown
2DS3	Unknown
2DS5	Unknown
3DS1	Unknown

associated HLA haplotype aids in diagnosis or prediction of disease predisposition.

The presence of a haplotype associated with disease is not diagnostic on its own, however. An example is the HLA-B27 type found in all cases of ankylosing spondylitis. HLA-B27 is also the most frequently found HLA-B allele. Many people, therefore, with the B27 allele do not have this condition. The laboratory is usually asked to perform HLA typing in persons showing disease symptoms or family histories of such disease to aid or confirm the diagnostic decisions.

Normal states are also controlled by multiple genetic and environmental factors. The genes for olfactory sensation, histone genes, genes encoding transcription factors and the butyrophilin (a constituent of milk in mammals) gene cluster are found in the MHC. The MHC may play a role, not only in the predisposition to disease, but protection from disease as well, including some infectious diseases, notably HIV.[68,69] In this role, certain HLA types are considered "protective".

Summary of Laboratory Testing

Standard HLA testing includes a range of methods and test designs (Table 15.8). Determining donor and recipient compatibility is the primary goal of pretransplant testing, especially for bone marrow transplant from unrelated donors. The extent of HLA matching will increase the prospect of successful transplantation with minimal GVHD. For example, the NMDP requests HLA typing results for HLA-A, B, and C class I antigens and DRB1, DQB1, and DPB1 class II antigens for prospective bone marrow donors.

Combinations of molecular tests, serum screening, and crossmatching further define acceptable HLA mismatches, and the results are used to prevent hyperacute (almost immediate) rejection in organ transplants. Identification of HLA types associated with disease also provides important information, especially for disease caused by multiple genetic and environmental factors. Finally, MLC, although not generally part of routine clinical testing, can predict cellular factors involved in rejection.

Laboratory results are key for selection of compatible donors, post-transplant evaluation, selection of optimal treatment strategy, and genetic disease predisposition. Molecular analysis has significantly increased the ease and ability to detect subtle differences in the MHC and associated regions. Nucleic acid analysis will play an expanding role in this area of laboratory analysis.

Advanced Concepts

HLA types are also associated with absence of inherited disease. Protection from genetic diseases is naturally enhanced by "hybrid vigor" or avoidance of inbreeding. The MHC may bring about such natural protection by control of mating selection to ensure genetic mixing. For example, studies with mice have shown that olfactory sensation (sense of smell) may play a role in mate selection. In these studies, female mice were able to distinguish male mice with H-2 alleles different from their own.[77] This supports the idea of selective pressure to mate with HLA-dissimilar partners to increase genetic diversity. Another study in humans showed that women found the scent of T-shirts worn by some male subjects pleasant and others unpleasant. The odors detected as pleasant were from HLA-dissimilar males. The women were apparently able to distinguish HLA types different from their own through sweat odors of male test subjects.[78]

Table 15.8 Summary of Testing for Pre- and Post-transplant Evaluation

Purpose	Test Design	Methods
Determine HLA type	Determine HLA types with standard references or by DNA sequence	Serology DNA-based typing
Determine serum antibody status	Serum screening against known HLA antibodies	CDC ELISA Flow cytometry
Crossmatching	Compare serum of recipient with that of prospective donors	CDC ELISA Flow cytometry
Determine T-cell mediated cytotoxicity between donor and recipient	Alloreactive T-cell characterization and quantitation	MLC Cytokine production

Case Study 15 • 1

A 50-year-old man complained of digestive disorders and what he presumed was allergy to several foods. He consulted his physician, who collected blood samples for laboratory testing. The man was in a high-risk group for certain diseases, notably celiac disease, which would produce symptoms similar to those experienced by this patient. A specimen was sent to the laboratory to test HLA-DQA and -DQB alleles. Almost all (95%) people with celiac disease have the DQA1*0501 and DQB1*0201 alleles, compared with 20% of the general population. A second predisposing heterodimer, DQ8, is encoded by the DQA1*0301 and the DQB1*0302 alleles. Most of the 5% of celiac patients who are negative for the DQ2 alleles display the DQ8 alleles. Serological results to detect the predisposing antigens, however, were equivocal. An SBT test was performed. The indicated alleles were detected by sequence analysis. The sequence results showed the following alleles at DQA1 and DQB1 loci:

DQA1*0501 DQB1*0201, DQB1*0401

Question: *Is it possible that this man has celiac disease based on his HLA haplotype?*

Case Study 15 • 2

A 43-year-old man consulted his physician about a lump on his neck and frequent night sweats. A biopsy of the mass in his neck was sent to the pathology department for analysis. An abnormally large population of CD20-positive lymphocytes was observed by morphological examination. Flow cytometry tests detected a monoclonal B-cell population with coexpression of CD10/CD19 and CD5. This population was 88% kappa and 7% lambda. The results were confirmed by the observation of a monoclonal immunoglobulin heavy chain gene rearrangement that was also monoclonal for kappa light chain gene rearrangement. The patient was initially treated with standard chemotherapy, but the tumor returned before the therapeutic program was completed. The tumor persisted through a second treatment with stronger chemotherapy plus local irradiation. Nonmyeloablative bone marrow transplant was prescribed. To find a compatible donor, the man's HLA type and the types of five potential donors were compared. HLA-A and -B types were assessed by serology, and HLA-DR type was determined by SSP-PCR and SSOP. The typing results are shown in the table.

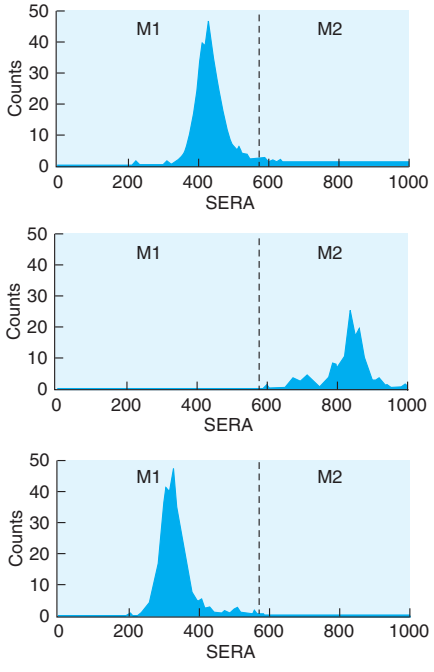
Recipient	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
A*0302	A*6601/04	A*2601	A*0302	A*3103/04	A*2610
A*2610	A*0302	A*4301		A*0309	A*0309
B*3906	B*0711	B*1528	B*3801	B*2702	B*3508
B*5307	B*5701	B*3919	B*3901	B*3501	
DRB1*0801	DRB1*0701	DRB1*1317	DRB1*0809	DRB1*0701	DRB1*1317
	DRB1*1317	DRB1*0422		DRB1*1117	DRB1*0701

Questions: *Which of the five donors is the best match for this patient? Which mismatches are acceptable? Using the donor bone marrow that matches most closely, is there more of a chance of graft rejection or GVHD?*

Case Study 15 • 3

A 19-year-old woman reported to a local clinic with painful swelling in her face. Routine tests revealed dangerously high blood pressure that warranted hospitalization. Further tests were performed, which led to a diagnosis of systemic lupus erythematosus. Due to complications of this disease, her kidney function was compromised, and she would eventually suffer kidney failure. With an alternative of life-long dialysis, a kidney transplant was recommended. Class I PRAs were assessed at 0% PRA for class I. An additional screen for class II PRA was performed by flow cytometry.

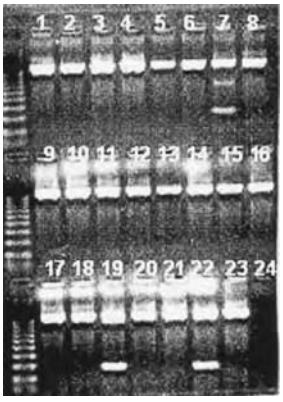
Class I and class II HLA typing was performed by SSP-PCR. An autocytotoxic crossmatch was also performed, revealing T-cell and B-cell positive antibodies, probably related to the lupus. The young woman's mother volunteered to donate a kidney to her daughter. Mother and daughter had matching blood group antigens and HLA-DR antigens, which are the most critical for successful organ transplant. The results from the typing tray are shown in the following figure:



Flow cytometry analysis of HLA class II antigens in recipient serum. Top = negative control; middle = positive control; bottom = patient serum.

(continued on following page)

(continued)



SSP-PCR results from an HLA-A typing tray for the daughter. Alleles were identified in lanes 7, 19, and 22.

SSP-PCR results for HLA-A, -B, and -DR and their serological equivalents are shown in the table.

Crossmatching of the daughter's serum and mother's cells was performed by cytotoxicity and flow cytometry. Both test results were negative.

Questions: *Is the mother a good match for the daughter? Based on the antibody and crossmatching studies, what is the risk of rejection? Before performing the HLA studies, how many of the daughter's antigens would be expected to match those of her mother?*

Daughter		**Mother**	
SSP-PCR	**Serology**	**SSP-PCR**	**Serology**
A*2419, A*3401	A24(9), 34(10)	A*1104, A*2419	A11, A24(9)
B*0802, B*5603	B8, B22	B*0804, B*5603	B8, B22
DRB1*0422, DRB1*1411	DR4, DR14(6)	DRB1*0422, DRB1*1317	DR4, DR13(6)

• STUDY QUESTIONS •

1. Which of the following is a high resolution HLA typing result?
   a. B27
   b. A*0202–0209
   c. A*0212
   d. A*2601/A*2605/A*26010/A*2615

2. Which of the following is a likely haplotype from parents with A25,Cw10,B27/A23,Cw5,B27 and A17, Cw4,B10/A9,Cw7,B12 haplotypes?
   a. A25,Cw10,B27
   b. A25,Cw5,B27
   c. A23,Cw4,B12
   d. A17,Cw4,B27

3. Upon microscopic examination, over 90% of cells are translucent after a CDC assay. How are these results scored according to the ASHI rules?

4. An HLA-A allele is a CTC to CTT (leu → leu) change at the DNA level. How is this allele written?
   a. HLA-A*02
   b. HLA-A*0201
   c. HLA-A2
   d. HLA-A*02N

5. A candidate for kidney transplant has a PRA of 75%. How will this affect eligibility for immediate transplant?

6. An SSOP probe recognizes HLA-DRB*0301–0304. Another probe recognizes HLA-DRB*0301/0304, and a third probe hybridizes to HLA-DRB*0301–0303. Test specimen DNA hybridizes to all except the third probe in a reverse dot blot format. What is the HLA-DRB type of the specimen?

7. What is the relationship between alleles HLA-A10 and HLA-A26(10)?

8. A CDC assay yields an 8 score for sera with the following specificities: A2, A28 and A2, A28, B7, and a 1 score for serum with an A2 specificity. What is the HLA-A type?

9. HLA-DRB1*1501 differs from DRB1*0101 by a G to C base change. If the sequence surrounding the base change is: …GGGTGCGGTTGCTGGAAAGAT… (DRB1*0101) or …GGGTGCGGTTCCTGGAAAGAT… (DRB1*1501), which of the following would be the 3′ end of a sequence-specific primer for detection of DRB1*1501?
   a. …ATCTTTCCAGGAACCC
   b. …ATCTTTCCAGCAACCC
   c. …ATCTTTCCAGC
   d. …ATCTTTCCAGG

10. The results of an SSP-PCR reaction are the following: lane 1, one band; lane 2, two bands, lane 3 no bands. If the test includes an amplification control multiplexed with the allele-specific primers, what is the interpretation for each lane?

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Chapter 16

Lela Buckingham

Quality Assurance and Quality Control in the Molecular Laboratory

OUTLINE

OBJECTIVES

- Describe proper specimen accession for molecular testing.
- Describe the optimal conditions for holding and storage of specimens and nucleic acid.
- Explain the basic components of molecular test performance, including quality assurance and controls.
- Discuss instrument maintenance, repair, and calibration, particularly for instruments used in molecular analysis.
- Describe recommendations for preparation and use of reagents in the molecular laboratory.
- Explain documentation and reporting of results, including gene sequencing results.

Congress passed the Clinical Laboratory Improvement Amendments (CLIA) in 1988 to establish quality testing standards to ensure consistent patient test results. CLIA specifies quality standards for proficiency testing, patient test management, quality control, personnel qualifications, and quality assurance for laboratories performing moderate- and/or high-complexity tests, including molecular testing. This chapter offers a brief overview of laboratory standards applied to molecular diagnostic tests.

Specimen Handling

Molecular tests, like any clinical laboratory tests, require optimal specimen handling and processing for accurate and consistent test results. The success of a test procedure is affected by the age, type, and condition of specimens. Therefore, specimen collection, transport, and handling in the laboratory require careful attention.

Preanalytical variables, both controllable and uncontrollable, must be taken into account for proper interpretation of test results. **Preanalytical error** is the consequence of erroneous or misleading results caused by events that occur prior to sample analysis. To minimize preanalytical error and maximize control of preanalytical variables, the Clinical and Laboratory Standards Institute (CLSI, formerly known as the National Committee for Clinical Laboratory Standards) provides recommendations for collection of specimens under standardized conditions.[1–3]

Each laboratory will have requirements for specimen handling, but general policies apply to all specimen collection. The condition of the specimen and, if necessary, the chain of custody is reviewed on receipt in the laboratory. If a specimen shows evidence of tampering or is otherwise compromised, the technologist must notify the supervisor. No specimen is accepted without proper labeling and identification on the specimen tube or container (placed by the person who collected the specimen), nor if the labeling on the specimen does not match that on the accompanying requisition. In addition to relevant patient identification, the test requisition includes the type of specimen material (e.g., blood or bone marrow), ordered test, date and time of collection, and a contact (pager or telephone number) of the ordering physician. When required (for molecular genetics or parentage testing), patient consent forms, ethnicity, photo identification of the individuals tested, and transfusion history or a pedigree may also be supplied with the test specimen. Forensic specimens may require a documented chain of custody. Bar-coding of this information expedites specimen accession and decreases the chance for error.

The laboratory should have written procedures for documentation of specimen accession. Accession books or electronic records are used to record the date of receipt, laboratory identifier, and pertinent patient information associated with the accession. If a specimen is unacceptable, the disposal or retention of the specimen is recorded in the patient report or laboratory quality assurance records. If not processed immediately, specimens are maintained in secure areas with limited access under the appropriate conditions for the analyte being tested (Fig. 16-1).

Molecular amplification methods have enabled laboratory professionals to perform nucleic acid-based testing on specimens with minimal cellular content, such as buccal cell suspensions and cerebrospinal fluids. These samples are centrifuged to collect the cells before DNA or RNA is extracted. For routine specimens tested by amplification methods, the entire specimen is often not used. In this case, or if more than one test is to be performed on the same specimen, care must be taken to avoid cross-contamination of specimens. This can most likely occur

Figure 16-1 Biohazard stickers are required for cabinets, refrigerators, or freezers that contain reagents or patient specimens.

from pipetting carryover. Moreover, an aliquot removed from a specimen is never returned to the original tube or vessel.

Hemoglobin inhibits enzyme activity. Specimens received in the laboratory should therefore be inspected for visual signs of hemolysis. Hemoglobin and coagulants are removed effectively in most DNA and RNA isolation procedures; however, if white blood cell lysis has also occurred, DNA or RNA yield will be reduced. This could result in false-negative results in qualitative testing or inaccurate measurements in quantitative analyses. Buffers such as BloodDirect (Novagen) and Extract-N-Amp (Sigma) or resins such as Chelex have been designed to sequester anticoagulants or hemoglobin for more rapid nucleic acid isolation without the inhibitory effects of these substances.

Solid tissues are best analyzed from fresh or frozen samples (Fig. 16-2), especially for Southern blot or long-range polymerase chain reaction (PCR) methods that require relatively high-quality (long, intact) DNA. Surgical specimens designated for molecular studies, if not processed immediately, should be snap-frozen in liquid nitrogen. This process both preserves nucleic acid and preserves gene expression patterns that may change upon tissue storage. Snap freezing is routinely performed in the surgical pathology laboratory as it is a common process for preserving tissue morphology for microscopic examination. Fixed, paraffin-embedded tissues generally yield lower quality DNA and RNA, depending on the type of fixative used, the amount of time of exposure of the tissue to the fixative, and how the specimen was handled prior to fixation. PCR and reverse transcription PCR (RT-PCR) amplification, however, are routinely performed on paraffin-embedded tissue samples. Methods such as Southern or Northern blot, requiring large fragments of DNA, are less likely to work consistently with fixed tissues.

Collection Tubes for Molecular Testing

Phlebotomy collection tubes are available with a number of different additives designed for various types of clinical tests. A selection of collection tubes commonly used for molecular biology studies is listed in Table 16.1. Some anticoagulants used in blood and bone marrow collection may adversely affect analytical results. Heparin has been shown to inhibit enzymes used in molecular analysis, such as reverse transcriptases and DNA polymerases in vitro.[4] The influence of this inhibition on molecular analysis is commonly accepted; however, heparinized samples have been processed successfully in many laboratories. The various experiences with heparin may reflect levels of resistance of enzymes from different sources. The assay design also has an effect. For instance, the inhibition of DNA polymerases compromises amplification of larger PCR products more than short ones. Due to the possible effects of heparin, trisodium EDTA (lavender top) or acid citrate dextrose (yellow top) tubes are recommended for most nucleic acid assays involving enzymatic treatment of the sample nucleic acid (Fig. 16-3).[5] High levels of disodium EDTA (royal blue capped tubes used for trace element studies) may also inhibit enzyme activity and should be avoided. One of the advantages of signal amplification methods such as bDNA technology is their decreased sus-

■ Figure 16-2 Tissue is received in the laboratory in fresh (left) or frozen (right) form. Fresh tissue may be supplied as a specimen on gauze or other substrate or in saline. The vial shown on the right contains tissue that was flash-frozen in isopentane. The vial is held immersed in liquid nitrogen, nitrogen vapors, or in a −70°C freezer.

Table 16.1 **A Selection of Collection Tubes**

Additive	Color	Nucleic Acid Testing
None	Red	Chemistry, serum, viral antibody studies
Sodium heparin (freeze-dried)	Green	Immunology, virology studies
Sodium heparin	Brown	Cytogenetic studies, molecular studies
Tripotassium EDTA (7.5%–15% solution)	Lavender	Virology, molecular biology studies
Acid citrate dextrose solution	Yellow	Molecular biology studies

ceptibility to chemical effects of anticoagulants. When a specimen is received in the laboratory, the anticoagulant is not usually noted in the accompanying documentation; the technologist should be aware of the type of collection tube used, especially if a specimen is received in heparin or other additive that may affect the test results.

In addition to the standard collection tubes, special collection tubes are designed particularly for stabilization of nucleic acids for molecular testing. These include the plasma preparation tubes (Vacutainer PPT, Becton Dickinson), which contain, in addition to the standard coagulants, a polymer gel that separates granulocytes and some lymphocytes from erythrocytes upon centrifugation. This type of tube is used for HIV and HCV analysis. The PPT tube is also used for separation of white cells for genetics and chimerism testing. The tube stoppers have a black "tiger" pattern over the appropriate anticoagulant color designation. Tubes such as the Tempus RNA Blood tube (Applied Biosystems) or the PAXgene Blood RNA tube (PreAnalytix) are designed to stabilize RNA. These tubes contain proprietary RNA stabilization agents that maintain the integrity of the RNA from collection through isolation.[6–8] Separated white blood cells from standard collection tubes can also be lysed in Trizol or TriReagent (Sigma) and the lysate stored at –70°C to stabilize RNA for several days. Stabilization of transcripts has become increasingly important, with more and more methods involving RNA transcript levels for measurement of gene expression. For example, quantitative RT-PCR analyses rely on transcript number to monitor the level of tumor cells or microorganisms. Serial analysis requires that the specimens received in the laboratory at different times be handled as consistently as possible. Immediate stabilization of the RNA is tantamount to having comparable results.[9] As an alternative to standard collection tubes, methods involving impregnated paper matrices have been proposed for nucleic acid analysis.[10,11]

Figure 16-3 For molecular analysis, blood or bone marrow specimens collected in EDTA or ACD tubes are preferred. Some methods are performed on serum collected in tubes without coagulant.

Precautions

Standard precautions are recommended by the Centers for Disease Control and Prevention for handling potentially infectious specimens. All specimens are potentially infectious, so they should all be handled with standard precautions using proper personal protective equipment (PPE) to prevent disease transmission. **Transmission-based** precautions including respirators are used with airborne or contact-transmissible agents. **Contact precautions** are designed for direct patient care where there is the potential for direct exposure to infectious agents on or from the patient. In general, standard precautions including gloves and gowns as PPE are used by the molecular laboratory technologist who has no direct contact with patients. Eye protection or masks are required in cases where frozen tissue is being processed or where spraying or splashing of sample may occur.

Gloves are highly recommended not only as part of standard precautions but also to protect nucleic acids from nuclease degradation (Fig. 16-4). Gloves are absolutely required for handling of RNA (Fig. 16-5). DNA is less susceptible to degradation from contaminating DNases; however, repeated handling of samples with-

■ Figure 16-4 Handling specimens with gloves is recommended to protect the technologist and to protect the sample nucleic acids from nucleases and other contaminants.

out gloves will adversely affect the integrity of the DNA over time. Standards and controls that are handled repeatedly are the most likely to be affected. Originally, having separate areas for DNA and RNA isolation was recommended. Some laboratories, however, isolate DNA and RNA on the same bench space. This requires maintenance of RNase-free conditions (see Chapter 4) for all nucleic acid isolation. In a common isolation arrangement such as this, care must be taken that if DNA samples are treated with RNase or RNA samples are treated with DNase that no inappropriate enzyme exposure occur.

■ Figure 16-5 Gloves are required for working with RNA.

Holding and Storage Requirements

Methods such as interphase and metaphase fluorescent in situ hybridization (FISH) and karyotyping require intact cellular structures or culture of cells so that only fresh specimens are acceptable. Many molecular methods, however, do not require integrity of tissue of cellular structure, only that the nucleic acid remains intact. In either case, circumstances may arise that require holding of specimens before analysis. DNA and RNA are stable when samples are collected and held under the proper conditions.[12,13] For example, multiple tests may be performed on snap-frozen specimens held at −70°C. Although most amplification methods are capable of successful analysis of limiting and challenging specimens, Southern or Northern blot methods will not work consistently on improperly handled specimens or nucleic acids stored under less than optimal conditions. The College of American Pathologists (CAP), which provides accreditation standards that are followed by CAP-accredited laboratories to improve the quality of their testing, has published recommendations for sample and isolated nucleic acid storage (Table 16.2).

For long-term storage, isolated nucleic acid is preferred. Conditions for storage of isolated nucleic acid have been recommended (Table 16.3). Cryotubes and specially designed labels are available for long-term nucleic acid storage at ultralow temperatures (Fig. 16-6).

The general rules for holding specimens for processing differ depending on the analyte and its stability in the cell. Written procedures indicate the proper handling of specimens for optimal performance of that procedure. Blood and bone marrow specimens sent to outside laboratories for molecular analysis can be shipped overnight at room temperature or with ice packs. Tissue is best shipped frozen on dry ice.

Isolated DNA of sufficient purity can be stored at room temperature for several months or at least 1 year in the refrigerator. Purified DNA can be stored at freezer temperatures (−20°C to −70°C) in tightly sealed tubes for up to 10 years or longer. Freezer temperatures are preferred for long-term storage; however, a clean DNA preparation in frequent use is better stored in the refrig-

Table 16.2 Specimen Storage Requirements for Nucleic Acid Extraction[33]

Nucleic Acid	Sample	Temperature	Time
DNA	Whole blood, buffy coat, bone marrow, fluids	22°–25°C	24 hours
		2°–8°C	72 hours*
		−20°C	At least 1 year*
		−70°C	More than 1 year*
	Tissue	22°–25°C	Not recommended
		2°–8°C	Up to 24 hours†
		−20°C	At least 2 weeks
		−70°C	At least 2 years
	Microorganisms in culture	22°–25°C	24 hours‡
		2°–8°C	72 hours‡
		−20°C	2–4 weeks‡
		−70°C	More than 1 year
	Cell lysates in GITC	22°–25°C	1–2 weeks§
RNA	Whole blood, buffy coat, bone marrow, fluids	22°–25°C	Not recommended§
		2°–8°C	2–4 hours*
		−20°C	2–4 weeks*
		−70°C	More than 1 year*
	Fluids collected in specialty RNA protection tubes	22°–25°C	5 days
		2°–8°C	7 days
		−20°C	2–4 weeks
		−70°C	At least 7 months
	Tissue	22°–25°C	Not recommended
		2°–8°C	Not recommended
		−20°C	Not recommended
		−70°C	At least 2 years
		−140°C (nitrogen vapor)	At least 2 years
	Cell lysates in GITC¶‖	22°–25°C	1–2 weeks§
	Cell lysates in RNA storage solution (Ambion)	22°–25°C	1 week
		2°–8°C	1 month
		−20°C	More than 1 year
	Microorganisms in culture	22°–25°C	24 hours‡
		2°–8°C	72 hours‡
		−20°C	2–4 weeks‡
		−70°C	More than 1 year

*Separation of white blood cells is recommended to avoid hemoglobin released upon hemolysis of red blood cells.

†Tissue types differ in stability and nuclease content.

‡Nucleic acid from cultured organisms is best isolated immediately on harvesting fresh cultures.

§ RNA status depends on the type of cell or tissue and the gene under study.[34]

‖ Depending on gene expression, adequate RNA may be isolated within a few hours. Storage of cell lysates in a stabilizing buffer is best for maintaining RNA.

¶GITC = guanidine isothiocyanate

Table 16.3 **Nucleic Acid Storage Requirements**[33]

Nucleic Acid	Matrix	Temperature	Time
DNA	TE* buffer or DNase-free water	22°–25°C	Up to 4 months
	Freeze-dried or dried on collection paper[35]	22°–25°C	More than 15 years
	TE buffer or DNase-free water	2°–8°C	1–3 years†
	TE buffer or DNase-free water	−20°C	At least 7 years
	TE buffer or DNase-free water	−70°C	More than 7 years
RNA	TE buffer or RNase-free (DEPC‡-treated) water	22°–25°C	Not recommended
	TE buffer or RNase-free (DEPC-treated) water	2°–8°C	Not recommended
	TE buffer or RNase-free (DEPC-treated) water	−20°C	Up to 1 month
	RNA storage solution (Ambion) §	−20°C	More than 1 month
	Ethanol	−20°C	More than 6 months
	TE buffer or RNase-free (DEPC-treated) water	−70°C	Up to 30 days
	Ethanol	−70°C	More than 6 months

*10 mM Tris, 1 mM EDTA, pH 8.0
†1 year for Southern blot
‡DEPC = diethyl pyrocarbonate
§1 mM sodium citrate, pH 6.4

erator so as to avoid DNA damage caused by multiple cycles of freezing/thawing. Shearing of DNA by freeze/thawing cycles can also occur in a frost-free freezer. As previously stated, PCR and methods that do not require large intact fragments of DNA are more forgiving with regard to the condition of the DNA.

Storage of isolated RNA at room temperature or refrigerator temperature is not recommended in the absence of stabilization. RNA suspended in ethanol can be stored at −20°C for several months. Long-term storage is best in ethanol at −70°C, although RNA suspended in diethylpyrocarbonate-treated water is stable for at least 1 month. As with DNA, the long-term survival of the RNA depends on the quality of the initial isolation and handling of the specimen.

Test Performance

Many tests in the molecular laboratory are individually designed or are adaptations based on published methods. Development of new tests in the clinical laboratory requires validation of the performance of the method and

Figure 16-6 Cryotubes with tight-fitting lids are recommended for long-term freezer storage of DNA and RNA.

Table 16.4 Measurements of Test Performance

Criteria	Definition	Example
Analytic sensitivity	Lower limit of detection of the analyte	The t(14;18) translocation test can detect 1 translocated cell in 10,000 normal cells, an analytic sensitivity of 0.01%.
Clinical sensitivity	Ability of test result to predict a clinical condition	95 of 100 patients with a gene mutation have a disease state, a clinical sensitivity of 95%.
Analytic specificity	Ability to detect only the analyte and not nonspecific targets	The Invader assay for factor V Leiden successfully detected mutations in 18 positive specimens while yielding negative results for 30 normal specimens (no false positives).
Clinical specificity	Disease-associated results only in patients who actually have the disease conditions	1 of 100 normal specimens displayed a gene mutation (1 false positive), a clinical specificity of 99%.
Precision	Reproducibility of test results	A quantitative method yields 99 results in agreement out of 100 runs, a precision of 99%.
Analyte measurement range	The range within which a specimen may be measured directly (without dilution or concentration)	A qPCR HSV assay yields reproducible linear results from 10 to 107 copies of HSV per 20 μL of CSF. Specimens within this range are measured directly.
Analytic accuracy	Production of correct results	99 of 100 specimens with mutations in the HCM gene are detected by sequencing with no mutations detected in normal specimens.

reagents in accurately detecting or measuring the analyte.[14] Test performance is assessed by several criteria (Table 16.4). These criteria are expressed as formulas.

The clinical sensitivity of an assay equals:

$$\frac{TP}{TP+FN} \times 100$$

The clinical specificity of an assay equals:

$$\frac{TN}{TN+FP} \times 100$$

The accuracy of an assay equals:

$$\frac{TN + TP}{TN+TP+FN+FP} \times 100$$

TN=true negative, TP=true positive, FN=false negative, FP=false positive.

These criteria are documented as part of the test validation process.

Test validation is performed on specimens of the types that will be encountered in the routine use of the test, such as frozen tissue, paraffin-embedded tissue, body fluids, and cultured cells. The number of specimens tested varies with the procedure and the availability of test material. Archived specimens are often used for this purpose. The results from the new test are compared with those of established procedures that may have been performed on these specimens or with the clinical diagnosis.[15–17] A standard form may be designed for preparation of reaction mixes by the test parameters determined in the validation process (Fig. 16-7).

Predeveloped and FDA-approved molecular methods are increasingly available. When these methods are incorporated, the test performance is verified by using the purchased reagent sets to test validation specimens. This verification establishes that the results of the commercial test performed in the individual laboratory are as predicted by the developer. If the commercial test is modified, validation is required to show equal or superior performance of the modified procedure.[18]

Once a procedure has been established, the method is documented in the laboratory according to CLSI guidelines.[19] The procedure description should include detailed information; for instance, primer and probe sequences, their purification conditions, and labeling. A copy of the standard form used to set up reaction mixes is included in the procedure description. A clear description of formulas and reporting units are required for quantitative results. Interpretation of qualitative data, acceptable ranges such as band patterns, product sizes, melting temperatures, and reasons for rejecting results are required informa-

PCR WORKSHEET (Ig heavy chain gene rearrangement)

Run #_____ Tech_____ Date_____

Tube #	Patient name	Specimen/ treatment	µg/µl	µl DNA*	µl H_2O*
1					
2					
3					
4					
5					
6					
7	Positive control				
8	Sensitivity control				
9	Negative control				
10	Reagent blank				

* Dilute sample to 1µg/µl in a volume of 15µl.

PCR reaction mixture for test primers

	(#rxns)
DNAse-free water	28.8µl x____ = ____µl
8 µM forward primer	2.5µl x____ = ____µl
8 µM reverse primer	2.5µl x____ = ____µl
1 mM dNTP	5.0µl x____ = ____µl
10x PCR buffer	5.0µl x____ = ____µl
750 µM $(NH_4)_2SO_4$	1.0µl x____ = ____µl
Taq polymerase	0.2µl x____ = ____µl

Place 45µl of mixture into PCR tubes. Add 5µl (5 µg) template to each PCR tube.

PCR mixture for β–globin control primers

DNAse water	30.8µl x____ = ____µl
2 µM β globin forward primer	1.25µl x____ = ____µl
2 µM β globin reverse primer	1.25µl x____ = ____µl
1 mM dNTP's	5.0µl x____ = ____µl
10x PCR buffer	5.0µl x____ = ____µl
50mM $MgCl_2$	1.5µl x____ = ____µl
Taq polymerase	0.2µl x____ = ____µl

Place 45µl of mixture into the PCR tubes. Add 5µl template to each PCR tube.

■ Figure 16-7 Example of a worksheet used to prepare PCR reaction mixes. A single reaction mix is made for multiple samples by multiplying the number of reactions by the volume of each reaction component and adding that amount to the master mix. Information regarding reagent lot numbers, PCR programs, and specimen dilutions may be included on the worksheet or documented separately.

tion. Methods used to score FISH or array results relative to internal control loci are also part of the written procedure. It is useful to incorporate pictures of gel patterns or instrument output data showing positive, negative, heterozygous, or other reportable results.

In the course of validation, the accuracy of a test will determine its correlation with disease, as performed in the testing laboratory. The indications for ordering the test are determined, based on the clinical utility as determined by the validation process, and documented in the procedure manual. For forensic testing, all aspects of the test from validation to test reporting should adhere to guidelines established by the DNA Advisory Board Standards and the Scientific Working Group on DNA Analysis Methods.[20]

The procedure manual or standard operating procedure is maintained in the laboratory and reviewed at least annually. If a test is discontinued, the written procedure, noted with the dates of initial use and retirement, is kept for at least 2 years. Some laboratory professionals maintain retired procedures for longer periods.

Controls

Controls are samples of known type or amount that are treated like and run with patient specimens.[21] Interpretation of test results always includes inspection of controls and standards to verify acceptable test performance. With qualitative tests, a **positive, negative** and, in some cases, a **sensitivity control** are required. The sensitivity control defines the lower limit of detection for more meaningful interpretation of negative results. These controls are sometimes called amplification controls when used with PCR techniques. Specifically, however, an **amplification control** is a target that should always amplify. The amplification control is used to distinguish true negative amplification results from false negatives resulting from amplification failure. In quantitative methods, **high positive, low positive**, and negative controls are included with each run. The high and low levels should be similar to critical points in the assay, such as the lowest detectable level of analyte.

Real-time PCR methods that automatically determine analyte levels require measurement of a **standard curve** or dilution series of analyte levels encompassing the levels expected from the patient specimens. On instruments, the standard curve must be run simultaneously with the specimens. In others, previously determined curves may be loaded into the software. Alternatively, results can be calculated manually by linear regression of the test results, using standard curve data in spreadsheet software.

In methods requiring detection of a target-specific product, or relative amounts of target, **internal controls** are run in the same reaction mix as the test specimen. For example, housekeeping genes are used as internal controls in methods quantifying infectious agents or detecting tumor cells by tumor-specific translocations. Centromere-specific probes serve as internal controls in FISH analyses as do housekeeping gene probes on microarrays. The presence of an internal control supplies a base for normalization of results. In PCR the internal control distinguishes false-negative results from failed amplifications (see Chapter 7). Internal controls that are amplified in the same tube with sample templates are designed to not interfere or inhibit target amplification, which could yield a false-negative result. Failed internal controls are documented and call for repeat of the assay.

The controls and standard curve should cover the critical detection levels or results of the method. Control results are continually monitored to spot trends or spikes outside of tolerance limits. Coefficients of variance or standard deviations of quantitative control levels should also be calculated at regular intervals. Laboratory professionals may establish criteria for control tolerance limits and document actions to be taken in the event of an unacceptable control result.

Controls are best prepared in larger quantities, aliquotted, and stored in conditions where they are most stable. Just as with new lots of other reagents, new aliquots are tested with old aliquots to verify consistent control results.

Quality Assurance

Periodic review and documentation of test results are required for all clinical testing, including molecular tests. Review might be, for example, in the form of rates of positive and negative results compared with expected rates from independent sources, such as published results, over time. This type of monitoring reveals trends or shifts in rates of positive or negative results. Critical values that require physician notification are established by validation and confirmed by monitoring.

As with other types of quantitative testing, molecular quantitative methods should have a defined dynamic range, sensitivity level, and accuracy.[22,23] For instance, a

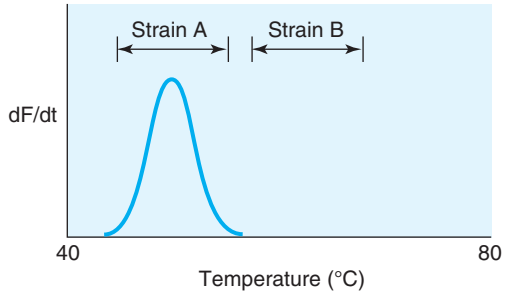


Figure 16-8 T_m ranges defined for two strains (A and B) of a theoretical microorganism are nonoverlapping. The melt curve shown would indicate that the test specimen contains strain A.

test that determines viral types by melt curves must include a defined narrow temperature range for the T_m of each viral type (Fig. 16-8). These values are established during the test validation and should be reviewed periodically. Test performance is monitored by inclusion of high, low, sensitivity, and negative standards in each run. Band patterns, melt curves, and peak characteristics should be defined with regard to how the results of the test are to be interpreted.

Assay levels that distinguish positive from negative results (**cut-off values**) must be well defined and verified at regular intervals. In assays such as single-strand conformation polymorphism (see Chapter 9), in which control patterns are not identical from run to run, normal controls for each scanned region are included in every run. Quantitative results should be within the linear range of the assay. The linear range is established by measuring dilutions or known concentrations of standard and establishing a direct correlation (standard curve) between test output and standard input. The technologist may observe that raw data are consistent with the final interpretation of the results. For example, if a viral load is interpreted as negative, the raw data should be below the cut-off value established for the test. Calculations and comparisons with standards used to verify test results should be described in the laboratory procedure manual.

Instrument Maintenance

Instruments used in the molecular laboratory must be monitored and maintained for consistent performance and accurate test results. Manufacturers supply recommendations for routine maintenance. Service contracts are used to provide support from trained service technicians. Laboratory professionals maintain a schedule and instructions for all routine maintenance, such as checking temperature settings, timers, and background levels. Parts are replaced as required or specified by the instrument manufacturer. Maintenance schedules should reflect the amount of use of the instrument. Most routine maintenance and minor troubleshooting, such as replacing bulbs or batteries, may be performed by the technologist with the aid of clear instructions from the manufacturer or as prepared by laboratory management. These instructions must be readily available to the technologist in the event of instrument malfunction. Technologists should be aware of the limits of user-recommended repairs and when service calls are indicated (Fig. 16-9). Laboratory professionals must document all maintenance, service calls, calibrations, and parts replacements.

Refrigerators and freezers used to store patient material and reagents are monitored at least daily (Fig. 16-10). Maximum/minimum thermometers register the highest and lowest temperature reached between monitoring points. During a busy shift, refrigerators and freezers may be opened frequently, causing the temperature to increase temporarily. This must be taken into account while monitoring. Out-of-range temperatures (e.g., more than $\pm 2^{\circ}C$ of the set temperature) are recorded. Confirmatory temperature checks at different times during the shift are required before further action is taken. Heat blocks, incubators, ovens, and water baths are also monitored for temperature stability and accuracy. U.S. National Institute of

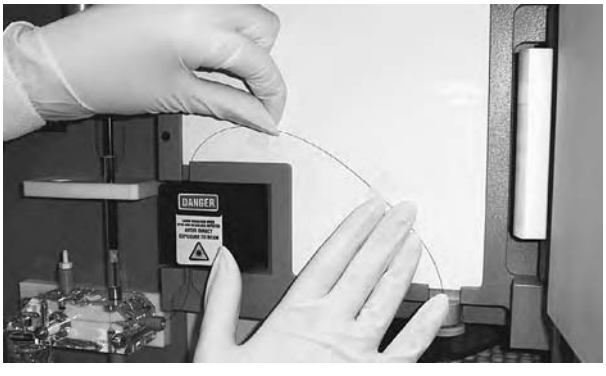


Figure 16-9 Routine maintenance, such as capillary replacement and instrument cleaning, is performed by the laboratory technologist. Dangerous or complex maintenance, such as repair or replacement of a laser source, is performed by the service representatives.

■ Figure 16-10 Certified chamber thermometers are used for monitoring temperatures in incubators, refrigerators, ovens, and freezers. The thermometers are supplied in plastic resin bottles containing liquid glycol.

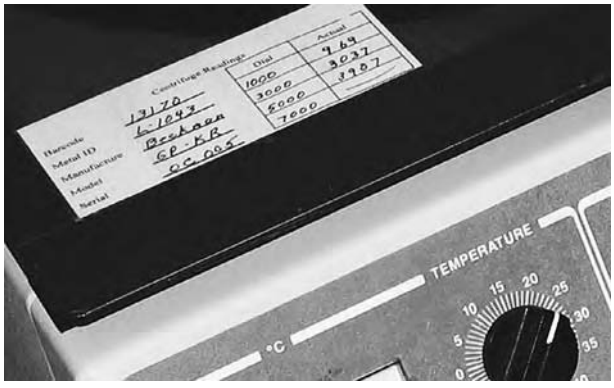
■ Figure 16-11 Block thermal cyclers may be monitored using a thermometer with a flexible probe. More thorough monitoring is performed with temperature probes and software that follows the ramping temperatures as well as the holding temperatures.

Standards and Technology (NIST)–certified standard thermometers are used for this type of monitoring. Thermometers verified by a NIST thermometer are also acceptable for this purpose. Specialized dry bath thermometers, which are encased in a 1.5-mL microfuge tube of mineral oil, are best for monitoring heat blocks. All storage and incubation equipment is kept clean and free of contaminated specimens and expired reagents. If out-of-date reagents are used for research or other purposes, they should be well marked and/or maintained in a separate area from the clinical test reagents.

Standard thermal cyclers, although they have no automated moving parts, decline in temperature control over time. This is especially true of block cyclers where hot and cold spots develop within the sample block. For this reason, thermal cyclers are checked periodically for proper temperature control. Thermometers with flexible probes (type K thermocouples) are convenient for checking representative wells in a block thermal cycler (Fig. 16-11). Approaches differ whether each well should be checked with each routine measurement or whether representative wells should be checked, with different wells checked each time. In some laboratories, test reactions are run in different wells to demonstrate successful amplification at each position on the block. More thorough and accurate temperature measurements are achieved with computer systems (e.g., Driftcon), with fixed or flexible probes designed to measure temperature in all wells throughout a PCR program, including ramping of the temperature up and down, overshooting set temperatures, and temperature drift during the hold phase of each step. Nonblock thermal cyclers, such as air-heated or modular instruments, are tested with probes modified to fit as the capillaries or tubes used in these instruments. Real-time thermal cyclers require additional maintenance of the detection system. Manufacturers supply materials for spectral calibrations. Background measurements are made using water or buffer samples. Each laboratory will establish the type and frequency of scheduled maintenance.

Centrifuges and microcentrifuges are monitored at least annually using a tachometer. In some institutions, technologists perform this calibration. Alternatively, an institutional engineering department may do it. The actual speed of rotation is determined and recorded along with the set speed or setting number on the centrifuge. This information is then posted on the instrument (Fig. 16-12).

Automatic pipettors used for dispensing specific quantities of reagents should be checked for accuracy before

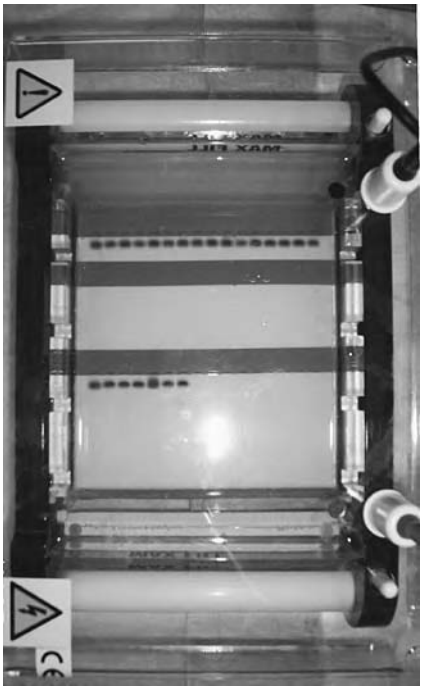


■ Figure 16-12 Centrifuge speeds are checked at least annually, and the results of actual and set speeds are posted on the instrument.

use and at 6-month intervals or as required according to use. Gravimetric methods, where measured samples of water are pipetted to a balance, have been used for many years.[24] The weights are converted to volumes. The mean of several measurements from the same pipet reveals its accuracy. The standard deviation or coefficient of variance is calculated to determine the degree of reproducibility (imprecision) of the pipet. Commercial pipet monitoring systems along with software are also available.[25] Alternatively, some laboratory professionals prefer to hire service providers who clean and check pipets on a per-pipet charge.

Electrophoresis power supplies are tested at least annually to ensure delivery of accurate voltage and current. Personnel should be trained in safe operation. Leads and connectors to gel baths should be free of precipitate (Fig. 16-13). This can be avoided by not leaving buffer in gel baths after electrophoresis runs. Capillary systems require cleaning of buffer and polymer delivery channels as well as replacing polymer at least twice per month. Capillaries should be replaced according to their suggested life span in number of uses. Temperature-controlled electrophoresis equipment, such as capillary systems and those used for constant temperature gel electrophoresis, is monitored for accurate temperature settings as recommended by the manufacturer.

Photographic equipment is frequently used in molecular laboratory procedures. Autoradiograms resulting from radioactive or chemiluminescent methods are developed in automated equipment or manually. The processing



■ Figure 16-13 Gel electrophoresis equipment must be maintained free of precipitate and properly handled to avoid shock exposure.

equipment is maintained with fresh fixing and developing solutions free of debris or sediment. Digital cameras are rapidly replacing Polaroid cameras for documenting gel data. Cameras should be firmly mounted and adjusted for optimal recording of gel data, free of shadows, dust, and other photographic artifacts (Fig. 16-14).

Periodically, background measurements are required on such instruments as fluorometric detectors (including real-time thermal cyclers), luminometers, and densitometers. Instrument manufacturers provide guidelines for acceptable background levels. Laboratory professionals may use these measurements or establish their own acceptable background levels. Corrective action, such as cleaning or filter adjustment, is documented in the laboratory maintenance records. Ultraviolet (UV) illuminators should be kept free of dust and properly shielded while in use. The technologists should keep track of the life span of the UV light source and replace it accordingly.

Spectrophotometers used to measure DNA, RNA, protein concentrations, and colorimetric assays and turbidity

■ Figure 16-14 Cameras should be mounted securely for gel photography. A digital camera, shown here, is mounted on a photographic hood. A mask shields the ultraviolet light source except for the area where the gel is illuminated.

■ Figure 16-15 Laminar flow hoods are used to protect against biological hazards and to help maintain a sterile environment.

must be checked annually or as recommended by the manufacturer. Maintenance includes scanning through the range of wavelengths used (e.g., 200–800 nm) with supplied materials or filters. Operation manuals will include instructions for calibration and maintenance.

Fume hoods and laminar flow (biological safety) hoods (Fig. 16-15) are monitored annually for proper air flow. Fume hood testing requires special equipment and is likely to be performed by building engineers. Laminar flow hoods are tested for proper filter performance and air displacement. This testing is performed at least annually or upon installation or movement of the hood. Professional service technicians or the hood manufacturers usually provide this type of certification.

For all detection systems, regular monitoring of functional characteristics will reveal any drift or trends that might affect test results. Tolerance limits should be established to warrant intervention by maintenance or recalibration of the instrument. Scheduled and unscheduled maintenance is documented and kept in the laboratory records. These records should be readily available to the technologists using the equipment.

Calibrations

Calibration is fitting an instrument or test system output with the actual concentration of a reference analyte by testing and making appropriate adjustments. In **calibration verification,** materials of known concentration throughout the reportable range are tested as patient samples to ensure the test system is accurate. If calibration verification fails, recalibration is required. CLIA-88 Regulations, 42CFR493.1255(b)(3), recommend performance of calibration verification at least every 6 months or when major components, instrument software, or lots of reagents of the test system are altered. Recalibration is also required if proficiency or other quality control testing fails or in the event of major instrument malfunction and repair. Manufacturers of test systems may also provide calibration schedules and instructions on how to perform calibrations. Laboratory professionals must verify calibration of systems performed by the manufacturer.

A variety of materials may be used for calibration, including previously tested specimens, reference standards, and proficiency testing material. There must be

independent assessment of the actual measurement of the calibration material. Once established, calibrator results should always be specified ranges of values. Calibration materials should cover at least three levels of measurement: low, medium, and high points. Analytes used for calibration should be in the same matrix (e.g., plasma or urine) as the patient specimens.[26] Calibrators are prepared and used separately from quality control standards (e.g., positive, negative, sensitivity controls) for routine runs.

Reagents

When reagents are replaced in a test method, the new lot is ideally tested on a previously positive and negative specimen as well as the run controls. Instructions on the preparation of reagents and the quantities used in each assay are included in the written laboratory protocol for each procedure. Lot numbers and working stocks of probes and primers used in amplification methods are documented and matched to test performance in the runs in which they were used. The sequences of primers and probes are also documented, as any sequence errors made during ordering or synthesis of the primers will adversely affect amplification specificity or even result in amplification failure. Probes used for linkage analysis and array technology are periodically updated as new markers are discovered so that probe sequences used for a given test should be recorded.

Primers are a critical component of PCR procedures. Primers are most conveniently supplied in lyophilized (freeze-dried) form from the DNA synthesis facility (Fig. 16-16). The supplier will also provide information on the quality, method of purification, molecular weight, and number of micrograms of dried primers. This information is used to rehydrate the primers to a stock solution concentration required for the PCR protocol. The resuspended primers are then diluted into working stocks.

Probes used for real-time PCR are supplied in solution; for example, a 100-μM stock solution that is diluted to 4- or 5-μM working stock before use in the procedure. When new working stocks are prepared (diluted from the probe stocks or resuspended primers), they are treated as new reagent lots. Master mixes of primers, probe, buffer, nucleotides, and enzyme may be prepared or purchased and used as working stock.

As is required for all reagents, instructions on preparation of primers, probes, and working stocks, along with

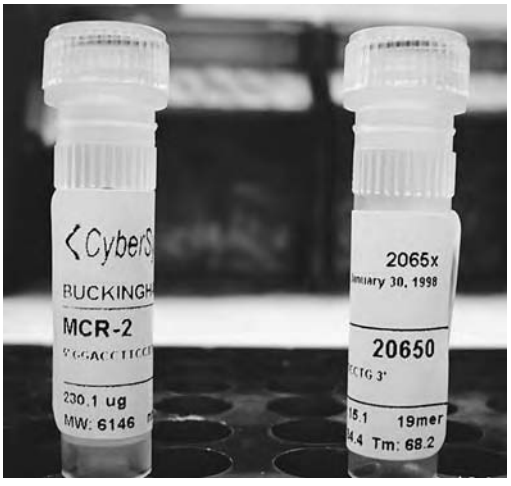


Figure 16-16 Primers are often purchased from DNA synthesis facilities. On receipt in freeze-dried form, the primers are easily resuspended in nuclease-free water or buffer to make a stock solution. The stock solution is then diluted into working stocks.

the sequences and binding sites of primers and the expected size of the amplicons, are documented as part of the written laboratory protocol. Polymorphisms or translocation breakpoints that affect primer binding should be noted in terms of the expected frequency in the population or in the number of successful amplifications.

For hybridization procedures, labeled probe solutions are treated as working stock and verified by parallel analysis with old lots. FISH probes are validated and verified according to recommended procedures.[18] The quality of new microarray lots is verified by the manufacturer or by hybridizing labeled nucleotides that bind to all probes on a representative array from the lot. RNA probes are maintained under RNase-free conditions to protect their integrity.

It is important to document descriptive information on probes used in the laboratory. This information includes the type of probe (genomic, cDNA, oligonucleotide, plasmid, or riboprobe) and the species of origin of the probe sequence. The sequence of the probe, a GenBank number or other identification of the target sequence or gene region recognized by the probe, and a restriction enzyme map of that region are also important information. Any known polymorphisms, sites resistant to endonuclease digestion, and cross-hybridizing bands should be noted. Recombination frequencies and map

positions must be documented for linkage procedures. For inherited disease tests, chromosomal location of the target gene and known mutant alleles and their frequencies in various ethnic groups might be cited in published reports. Labeling methods and standards for adequacy of hybridization are included in the test procedure manual.

In multiplex reactions, primer and/or probe competition for substrates may affect results. Multiple fluorochrome signals in fluorescence assays may also cross into each other's detection ranges. For gel or capillary sizing, products of multiplex reactions should be reasonably different so that banding patterns do not complicate interpretation. For example, multiplex STR analysis by PCR includes 13 sets of primers that produce 13 amplicons labeled with three different fluorochromes (see Chapter 11). The range of sizes of each amplified STR locus is designed not to overlap others labeled with the same fluorochrome. The instrument that detects the fluorescence is also calibrated to subtract any overlap of detection of one fluorochrome with another.

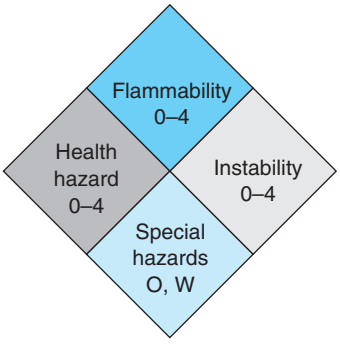
Analyte-specific reagents (ASR) are probes, primers, antibodies, and other test components that detect a specific target, such as a cell surface protein or DNA mutation. ASRs comprise the active part of "home brew" tests. ASRs are usually purchased from an outside manufacturer. ASRs are classified as I, II, or III. Most ASRs used in the molecular laboratory are class I. Several molecular tests are available as ASRs in infectious disease, tissue typing, and other areas of molecular diagnostics. Approved molecular methods include tests that utilize FISH, Hybrid Capture, PCR, and microarray technologies. Class II and III ASRs include those used by blood banks to screen for infectious diseases and those used in diagnosis of certain contagious diseases such as tuberculosis. Class I ASRs are not subject to special controls by the U.S. Food and Drug Administration. The test performance of class I ASRs is established during test validation.

Chemical Safety

Volatile and flammable reagents are stored in properly vented and explosion-proof cabinets or refrigeration units (Fig. 16-17). The National Fire Protection Association (NFPA) has developed a series of warning labels for universal use on all chemical containers (Fig. 16-18).[27] Secondary or reinforced containers are required for transport and handling of dangerous chemicals, such as concentrated acids and phenol.

■ Figure 16-17 Flammable and explosive materials are stored in designated protective cabinets or explosion-proof refrigerators.

Radioactive chemicals are used in some molecular methods (Table 16.5). Although methods involving radiation are increasingly being replaced by nonradioactive alternatives, some laboratory procedures still use these agents. The Nuclear Regulatory Commission requires that laboratory personnel working with radioactive



■ Figure 16-18 NFPA hazard labels have three parts, labeled with numbers 0 to 4, depending on the amount of hazard, from none (0) to severe (4). The fourth section has two categories. O indicates a strong oxidizer, which greatly increases the rate of combustion. The stricken W symbol indicates dangerous reactivity with water, which would prohibit the use of water to extinguish a fire in the presence of this chemical.

Table 16.5 **Examples of Radionuclides Used in Laboratory Methods**

Radioisotope	Half-Life*	Radiation (MeV)†	Travel in Air	Critical Organs
^{32}P	14.29 days	β, 1.709	20 feet	Bone, whole body
^{33}P	25.3 days	β, 0.249	20 feet	Bone, whole body
^{3}H	12.35 years	β, 0.019	0.65 inches	Body water
^{14}C	5730 years	β, 0.156	10 inches	Whole body, fat
^{35}S	87.39 days	β, 0.167	11 inches	Whole body, testes
^{125}I	60.14 days	X,γ, 0.035	3 feet	Thyroid

*Time for half of the radiation emission to dissipate
†Million electron volts

reagents maintain a radiation safety manual providing procedures for the safe handling of radioactive substances in both routine and emergency situations. The Occupational Safety and Health Administration (OSHA) has also developed regulations regarding ionizing and nonionizing radiation.

Radioactive reagents and methods are performed in designated areas. Working surfaces are protected with absorbent paper, drip trays, or other protective containers. Potentially volatile radioactive materials are handled under a fume hood. Radioactive waste is discarded in appropriate containers, separate from normal trash, according to regulations. Some isotopes with short half-lives may be stored over approximately seven half-lives, checked for residual emissions, and then discarded with regular waste. Containers and equipment used in these areas should be labeled with "Caution Radioactive Material" signs (Fig. 16-19). Signs should be posted on the rooms where radioactive materials are used. OSHA has specifications for accident prevention signs and tags for radiation and other occupational hazards.

Figure 16-19 Rooms, cabinets, and equipment containing radioactive chemicals are identified with radiation safety labels.

Laboratory personnel working with radioactive material should receive special training for safe handling, decontamination, and disposal of radiation. Laboratory instructions for working with radiation should include inspection and monitoring of shipments as required by the U.S. Department of Transportation. Work spaces are decontaminated daily and checked at least monthly by swipe testing or by Geiger counter. Technologists wear gloves, lab coat, and safety glasses when handling radioactive solutions. Radiation badges are worn when handling 1.0 mCi or more. Exposure increases with decreasing distance from the radioactive reagent (see Table 16.5), so exposure at close distance, such as working over open containers, should be avoided. For isotopes such as ^{32}P, acrylic shielding is required for work, storage, and waste areas (Fig. 16-20).

Proficiency Testing

Proficiency testing refers to the analysis of external specimens from a reference source supplied to independent laboratories.[28] Proficiency testing is performed to assess the skills (competency) of laboratory personnel performing molecular assays as well as the performance of the assay itself. Availability of comprehensive test specimens in the rapidly expanding area of molecular diagnostics is sometimes problematic. The CAP supplies specimens for molecular oncology, engraftment, and microsatellite instability testing among others (www.cap.org). A number of analytes, however, are not available, especially for tests that are offered in a small number of laboratories. If proficiency specimens are not commercially available, laboratories can exchange blinded split specimens; alternatively, blinded specimens measured or documented by independent means such as chart review can be tested

Figure 16-20 Acrylic shielding is required for working with gamma emitters, such as ^{32}P and ^{33}P.

within the laboratory.[29,30] If at all possible, interlaboratory testing is preferable.

Proficiency testing is performed at least twice a year, with the proficiency samples tested within routine patient runs. The specific procedures should be defined and documented in the laboratory. Errors or incorrect responses for proficiency specimens are documented along with the corrective action taken, if necessary.

Documentation of Test Results

Test results in the form of electropherograms, gel images, and autoradiograms should be of sufficiently high quality that results are unequivocal. This includes clear bands or peaks without high background, cross hybridization, distortions, and other artifacts. Controls should also be clear and consistent and reflect the expected size or level. Molecular weight ladders on gels, autoradiograms, or electropherograms should cover the expected range of band or peak sizes produced from the specimen. For example, if primers used to detect a t(14;18) translocation test by PCR yield amplicons expected to range 150–500 bp, the molecular weight ladder used must range from less than 150 bp to more than 500 bp.

A record of the assay conditions and reagent lot numbers is kept with patient results. Identification of the technologist performing the assay may also be included. Documentation of quality and quantity of the isolated DNA or RNA is also required, especially if designated amounts of nucleic acids are used for an assay. Quantity and quality are documented in the form of spectrophotometry or fluorometry data or quantity or gel photographs of high molecular weight DNA or ribosomal RNA quality (see Chapter 4). The quality of RNA analyzed by Northern blot or RT-PCR may be assessed by monitoring a housekeeping gene, ribosomal RNA expression, or other calibrator.

If DNA is cut with restriction enzymes for Southern blot or PCR-restriction fragment length polymorphism, complete cutting by the restriction enzyme is verified on control targets and documented by photography of the cut DNA on the gel after electrophoresis. DNA digested with DNase for array analysis is also documented in this way to confirm proper fragment sizes. If specimen nucleic acid is labeled for hybridization arrays, labeling efficiency is assessed by measurement of the specific activity (signal per ng nucleic acid). For Southern blots, patient identification, gel lane (well) number, and probe target and type are also documented. It is also recommended that the test documentation should include prehybridization and hybridization conditions and probe and hybridization buffer lot numbers.

In situ results, such as FISH, are correlated with histological findings (stained sections) of tissue morphology. This is required when the molecular target detection is significant in specific cells; for example, with p53 detection in tumor cells. Documentation includes images of at least one normal cell with at least two abnormal cell results. These images are cross-referenced or retained together with photographs, films, and autoradiographs generated from additional testing of the same specimen. All these records are labeled with patient identification, sample numbers, run identifiers, and the date of assay.

All of the raw data are retained with the final report and clinical interpretation of the test results. Careful documentation is important because molecular diagnostic results may differ from results from other laboratories or from the clinical diagnosis. Such discrepancies occur most often with amplification methods because of their high sensitivity. If a molecular result is questioned, investigation of the discrepancy includes review of the raw

data. Results of this investigation along with any corrective action taken are noted in the laboratory records.

Gene Sequencing Results

Direct sequencing is increasingly used in clinical applications to detect gene mutations or to type microorganisms. Sequence data must be of adequate quality with acceptably low baseline, especially if heterozygous target states are to be detected. Each nucleotide peak or band should be unequivocal. Sequencing should be performed on both complementary strands of the template to confirm sequenced mutation or type. Repeated sequencing across the same area, or **resequencing**, for known sequence changes is sometimes performed only on one template strand; however, sequencing of both strands is best.

Criteria for acceptance of sequencing data include correct assignment of the nucleotide sequence in a defined region surrounding the critical area, not including the amplification primer binding sites. Furthermore, a specified level of band or peak quality (intensity or fluorescence levels, respectively) with reasonably low background is assigned. Defined limits of fluorescence ratios are set to identify true heterozygous base positions. Ideally, a heterozygous position will have equal fluorescence contribution from the two genotypes, and the peak height will be approximately half that of a homozygous genotype at that position. Results are expressed in the standard nomenclature for DNA or protein sequences (see Chapter 9).

The utility of sequence data requires published normal or type-specific sequences. In the case of gene mutations, electronic or published databases of known mutations and polymorphisms are available for frequently tested genes. These records, especially Internet databases, are updated regularly. Newly discovered mutations are classified according to the type of mutation; the laboratory director or consultant uses published guidelines to determine if the mutation is clinically significant.[31] For example, a silent mutation will not affect protein function, whereas a frameshift mutation will.

Reporting Results

Test results are reported in terms that are unambiguous to readers who may not be familiar with molecular methods or terminology. The test report must clearly convey the method or manufactured kit used, the locus, mutation or organism tested, the analytical interpretation of the raw data, and the clinical interpretation of the analytical result. This interpretation includes the penetrance of mutations; that is, the probability of having a mutation but not getting the associated disease.

The likelihood of false-positive or false-negative results are also included in a report. Mutation detection is not guaranteed, especially in large genes with hundreds of possible mutations that may or may not effectively compromise gene function. The mutation detection rate for this type of gene and the residual risk of undetected mutations are therefore included in the test report. Negative results from tests for specific point or chromosomal mutations are reported in terms of the sensitivity of the test, e.g., less than 0.01% chance of mutation, or, alternatively, negative accompanied with the sensitivity levels of the test. For parentage reports, the combined paternity index, the probability of paternity as a percentage, prior probability of paternity used in calculations, and the population used for comparison are reported.

The laboratory director, pathologist, or other clinical expert reviews the analytical interpretation, determines the clinical interpretation, and verifies the final results with an actual or electronic signature on the test report. An internal laboratory summary sheet is often useful for compiling pertinent information (Fig. 16-21). Test results should not be released before they are reviewed by the director. Molecular diagnostic tests, in particular, may have technical complexities that influence the meaning of the test result. These results are best communicated with the clinical significance of the laboratory findings.

When class I ASRs are used in an analytical method, the following disclaimer is included in the test report: "The FDA has determined that such clearance or approval is not necessary. This test is used for clinical purposes. It should not be regarded as investigational or for research. This laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA-88) as qualified to perform high complexity clinical laboratory testing."[32]

The disclaimer is not required for tests using reagents that are sold together with other materials or an instrument as a kit nor for reagents sold with instructions for use.

Confidentiality of molecular test results is essential. All results, and particularly molecular genetic results, may affect insurability, employment, or other family members. Results are released only to the ordering physi-

PATIENT SAMPLE STORAGE AND RESULT SUMMARY

Date:

Patient ID Label Sex: Diagnosis:

DOB: Specimen:

Age: Surgical Path #:

I.
Flow Cytometry Accession # _____ Date of Procedure:_____

Genetics Accession #_____ Date of Procedure:_____

Bone Marrow Aspirate #_____ Date of Procedure:_____

II. Tissue Storage: Freezer Box #_____ RNA Storage Box #_____Volume_____

III. RNA Isolation

O.D. Value	260/280	Volume	Concentration

IV. DATA: RUN #_____

Previous Results:

Controls Acceptable: yes/no

__ Positive for BCR/ABL Translocation at M-bcr b2/a2 fusion
__ Positive for BCR/ABL Translocation at M-bcr b3/a2 fusion
__ Positive for BCR/ABL Translocation at m-bcr e1/a2 fusion
__ Negative for BCR/ABL Translocation

Pathology Result_____ Cytogenetics Result_____

V. Interpretation of lab results:_____
_____
_____
_____

Remarkable case:_____
_____

Verbal report to:_____Date_____By_____

VI. Physician's Interpretation:_____
_____
_____

Reviewed:_____ Date_____

■ Figure 16-21 Example of a patient result summary sheet used for documentation of test results of a BCR/ABL analysis by RT-PCR. Information included will differ depending on the disease and the type of test.

cian or other authorized personnel such as genetic counselors or nurse coordinators. Technologists should refer requests for patient data to supervisors. Data sent by facsimile must be accompanied by a disclaimer such as: "The documents accompanying this telecopy transmission contain confidential information belonging to the sender that is legally privileged. This information is intended only for the use of the individual or entity named above. The authorized recipient of this information is prohibited from disclosing this information to any other party and is required to destroy the information after its stated need has been fulfilled. If you are not the intended recipient, you are hereby notified that any disclosure, copying, distributing, or action taken in reliance on the contents of these documents is strictly prohibited. If you have received this telecopy in error, please notify the sender immediately to arrange for return or destruction of these documents."

Test results are not released to employers, insurers, or other family members without the patient's expressed consent. Any data discussed in a public forum are presented such that no patient or pedigree is identifiable by the patient or the general audience. Written consent from the patient may be required under some circumstances. Each institution will have a department that oversees the lawful use of confidential information.

Technologists working in the area of molecular pathology will encounter tests in which the final details are determined empirically. As a result, a test procedure may differ from one laboratory to another. Even after the test procedure is established, troubleshooting is sometimes required as the procedure is put to use on a routine basis. Some reactions that work well for short-term research may prove to be less consistent and reproducible than is required in the clinical laboratory setting.

Biotechnology is fast developing standard reagent sets and instrumentation for the most popular tests, but these also differ from one supplier to another. Furthermore, due to market demands, test reagent kits may be modified or discontinued. If replacement reagents are available, they may not be identical to those previously used. Ongoing tests then have to be optimized. This can be a concern where turnaround times are critical.

It then becomes the responsibility of the technologist to perform and monitor tests on a regular basis to maintain consistency and accuracy of results. The technologist who understands the biochemistry and molecular biology of these tests will be better able to respond to these problems. In addition, with the quickened evolution of the sciences, a knowledgeable technologist can better recognize significant discoveries that offer potential for test improvement.

• STUDY QUESTIONS •

What actions should be taken in the following situations?

1. An unlabeled collection tube with a requisition for a factor V Leiden test is received in the laboratory.
2. After PCR, the amplification control has failed to yield a product.
3. An isolated DNA sample is to be stored for at least 6 months.
4. A bone marrow specimen arrives at the end of a shift and will not be processed for the Bcl2 translocation until the next day.
5. The temperature of a refrigerator set at 8°C (±2°C) reads 14°C.
6. A PCR test for the BCR/ABL translocation was negative for the patient sample and for the sensitivity control.
7. A fragile X test result has been properly reviewed and reported.
8. A bottle of reagent alcohol with a 3 in the red diamond on its label is to be stored.
9. The expiration date on a reagent has passed.
10. Test results are to be faxed to the ordering physician.

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CHAPTER 1

1. What is the function of DNA in the cell?
   Storage of genetic information

2. Compare the structure of the nitrogen bases. How do purines and pyrimidines differ?
   Purines have a double ring; pyrimidines have a single ring.

3. Write the complementary sequence to the following:
   5′AGGTCACGTCTAGCTAGCTAGA3′
   3′TCCAGTGCAGATCGATCGATCT5′

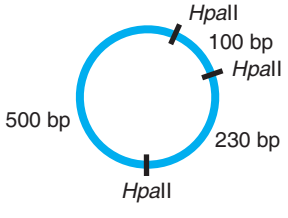
4. Which of the ribose carbons participates in the phosphodiester bond?
   5′ carbon, 3′ carbon

5. Which of the ribose carbons carries the nitrogen base?
   1′ carbon

6. Why does DNA polymerase require a primer?
   A 3′ hydroxyl group from an existing nucleotide must be present to form the phosphodiester bond.

Restriction Enzyme Analysis

1. A plasmid was digested with the enzyme *Hpa*II. On agarose gel electrophoresis, three bands are observed: 100, 230, and 500 bp.
   a. How many *Hpa*II sites are present in this plasmid?
      3
   b. What are the distances between each site?
      100 bp, 230 bp, 500 bp
   c. What is the size of the plasmid?
      830 bp
   d. Draw a picture of the plasmid with the *Hpa*II sites.



A second cut of the plasmid with *Bam*H1 yields two pieces, 80 and x bp.
   e. How many *Bam*H1 sites are in the plasmid?
      2
   f. What is x in base pairs (bp)?
      830 bp − 80 bp = 750 bp

2. How would you determine where the *Bam*H1 sites are in relation to the *Hpa*II sites?
   Cut the plasmid with both enzymes at the same time.

3. The plasmid has one *Eco*R1 site into which you want to clone a blunt-ended fragment. What type of enzyme could turn an *Eco*R1 sticky end into a blunt end?
   5′ to 3′ single-strand exonuclease

Recombination and DNA Transfer

1. Compare how DNA moves from cell to cell by a) conjugation, b) transduction, and c) transformation.
   a) Cell-to-cell contact
   b) Viral or bacteriophage vectors
   c) Fragmented or plasmid DNA

CHAPTER 2

RNA Secondary Structure

1. Draw the secondary structure of the following RNA. The complementary sequences (inverted repeat) are underlined.
   5′CAUGUUCAGCUCAUGUGAACGCU3′

2. Underline the rest of the *two* inverted repeats in the following RNA, then draw the secondary structure.
   5′CUGAACUUCAGUCAAGCAUGCACUGAUGCUU3′

OK as set, same as Right Running head

The Lac Operon

1. Using the depiction of the Lac operon in Figures 2-9 and 2-10 indicate whether gene expression (transcription) would be on or off under the following conditions:
   (P = promoter; O = operator; R = repressor)
   a. P+ O+ R+, no inducer present
      OFF
   b. P+ O+ R+, inducer present
      ON
   c. P- O+ R+, no inducer present
      OFF
   d. P- O+ R+, inducer present
      OFF
   e. P+ O- R+, no inducer present
      ON
   f. P+ O- R+, inducer present
      ON
   g. P+ O+ R-, no inducer present
      ON
   h. P+ O+ R-, inducer present
      ON
   i. P- O- R+, no inducer present
      OFF
   j. P- O- R+, inducer present
      OFF
   k. P- O+ R-, no inducer present
      OFF
   l. P- O+ R-, inducer present
      OFF
   m. P+ O- R-, no inducer present
      ON
   n. P+ O- R-, inducer present
      ON
   o. P- O- R-, no inducer present
      OFF
   p. P- O- R-, inducer present
      OFF

Epigenetics

1. Indicate whether the following events would increase or decrease expression of a gene:
   a. Methylation of cytosine bases 5′ to the gene
      Decrease
   b. Histone acetylation close to the gene
      Increase
   c. siRNAs complementary to the gene transcript
      Decrease

CHAPTER 3

1. Indicate whether the following peptides are hydrophilic or hydrophobic?
   a. MLWILSS
      Hydrophobic
   b. VAIKVLIL
      Hydrophobic
   c. CSKEGCPN
      Hydrophilic
   d. SSIQKNET
      Hydrophilic
   e. YAQKFQGRT
      Hydrophilic
   f. AAPLIWWA
      Hydrophobic
   g. SLKSSTGGQ
      Hydrophilic

2. Is the following peptide positively or negatively charged at neutral pH?
   GWWMNKCHAGHLNGVYYQGGTY
   Positive

3. Consider an RNA template made from a 2:1 mixture of C:A. What would be the three amino acids *most* frequently incorporated into protein?
   Proline, histidine, threonine

4. What is the peptide sequence encoded in AUAUAUAUAUAUAUA…?
   IYIYIYIYIY…

5. Write the *anticodons*, 5′ to 3′, of the following amino acids:
   a. L
      UAA, CAA, AAG, GAG, UAG, CAG
   b. T
      AGU, GGU, UGU, CGU
   c. M
      CAU

d. H
AUG, GUG
e. R
ACG, GCG, UCG, CCG, UCU, CCU
f. I
AAU, GAU, UAU

6. A protein contains the sequence LGEKKWCLRVNPKGLDESKDYLSLKSKYLLL. What is the likely function of this protein?
This protein has a leucine zipper and it is likely to be a transcription factor.

7. A histone-like protein contains the sequence: PKKGSKKAVTKVQKKDGKKRKRSRK. What characteristic of this sequence makes it likely to associate with DNA?
This protein has a lot of lysines. The resulting positive charges would bind favorably to the negatively charged DNA.

CHAPTER 4

DNA Quantity/Quality

1. Calculate the DNA concentration in μg/mL from the following information:
   a. Absorbance reading at 260 nm from a 1:100 dilution = 0.307.
   0.307 Abs × 50 (μg/mL) × 100 = 1535 μg/mL
   b. Absorbance reading at 260 nm from a 1:50 dilution = 0.307.
   0.307 Abs × 50 (μg/mL) × 50 = 767.5 μg/mL
   c. Absorbance reading at 260 nm from a 1:100 dilution = 0.172.
   0.172 Abs × 50 (μg/mL) × 100 = 860 μg/mL
   d. Absorbance reading at 260 nm from a 1:100 dilution = 0.088.
   0.088 Abs × 50 (μg/mL) × 100 = 440 μg/mL

2. If the volume of the above DNA solutions was 0.5 mL, calculate the yield for a. to d.
1a. 1535 μg/mL × 0.5 mL = 767.5 μg
1b. 767.5 μg/mL × 0.5 mL = 383.75 μg
1c. 860 μg/mL × 0.5 mL = 430 μg
1d. 440 μg/mL × 0.5 mL = 220 μg

3. Three DNA preparations have the following Abs_{260} and Abs_{280} readings:

Sample No.	Abs_{260}	Abs_{280}
1	*0.419*	*0.230*
2	*0.258*	*0.225*
3	*0.398*	*0.174*

For each sample 1-3, based on the Abs_{260}/Abs_{280} ratio, is each preparation suitable for further use? If not, what is contaminating the DNA?
1 Abs_{260}/Abs_{280} = 0.419/0.230 = 1.82, suitable
2 Abs_{260}/Abs_{280} = 0.258/0.225 = 1.14, unsuitable, protein contamination
3 Abs_{260}/Abs_{280} = 0.398/0.174 = 2.28, may be suitable, RNA contamination

RNA Quantity/Quality

1. Calculate the RNA concentration in μg/mL from the following information:
   a. Absorbance reading at 260 nm from a 1:100 dilution = 0.307
   0.307 Abs × 40 (μg/mL) × 100 = 1228 μg/mL
   b. Absorbance reading at 260 nm from a 1:50 dilution = 0.307
   0.307 Abs × 40 (μg/mL) × 50 = 614 μg/mL
   c. Absorbance reading at 260 nm from a 1:100 dilution = 0.172
   0.172 Abs × 40 (μg/mL) × 100 = 688 μg/mL
   d. Absorbance reading at 260 nm from a 1:100 dilution = 0.088
   0.088 Abs × 40 (μg/mL) × 100 = 352 μg/mL

2. If the volume of the above RNA solutions was 0.5 mL, calculate the yield for a. to d.
1a. 1228 μg/mL × 0.5 mL = 614 μg
1b. 614 μg/mL × 0.5 mL = 307 μg

1c. 688 μg/mL × 0.5 mL = 344 μg
1d. 352 μg/mL × 0.5 mL = 176 μg

3. An RNA preparation has the following absorbance readings:
   Abs_{260} = 0.208
   Abs_{280} = 0.096
   Is this RNA preparation satisfactory for use?
   Abs_{260}/Abs_{280} = 0.208/0.096 = 2.17, suitable

CHAPTER 5

1. You wish to perform a resolution of your restriction enzyme–digested DNA fragments. The size of the expected products ranges 500–100 bp. You discover two agarose gels polymerizing on the bench. One is 5% agarose. The other is 2% agarose. Which one might you use to resolve your fragments?
   2%

2. After completion of the run of fragments along with the proper molecular weight standard on the agarose gel, suppose a. or b. was observed. What might be explanations for these? (Assume you have included a molecular weight marker in your run.)
   a. The gel is blank (no bands, no molecular weight standard).
      Samples were not loaded properly.
      Electrodes were switched.
      Gel was not stained properly.
   b. Only the molecular weight standard is visible.
      Samples were not loaded properly.
      Samples were diluted, degraded, or otherwise compromised.
      Gel was not stained evenly.

3. How does PFGE separate larger fragments more efficiently than standard electrophoresis?
   Repeated reorientation forces larger fragments through the gel matrix more effectively.

4. A 6% solution of 19:1 acrylamide is mixed, deaerated, and poured between glass plates for gel formation. After an hour, the solution is still liquid. What might be one explanation for the gel not polymerizing?
   No catalyst was added.

5. A gel separation of RNA yields aberrantly migrating bands and smears. Suggest two possible explanations for this observation.
   Poor RNA sample quality.
   Inadequate denaturation of the RNA before loading.

6. Why does DNA not resolve well in solution (without a gel matrix)?
   The size and charge of DNA have opposing effects on migration.

7. Why is SyBr green less toxic than EtBr?
   SyBr green binds in the minor groove of DNA, unlike EtBr, which intercalates between the bases. Intercalation by EtBr is more likely to cause DNA mutations.

CHAPTER 6

1. Calculate the melting temperature of the following DNA fragments using the sequences only:
   a. AGTCTGGGACGGCGCGGCAATCGCA
      TCAGACCCTG CCGCG CCGTTAGCGT
      84°C
   b. TCAAAAATCGAATATTTGCTTATCTA
      AGTTTTTAGCTTATAAACGAATAGAT
      64°C
   c. AGCTAAGCATCGAATTGGCCATCGTGTG
      TCGATTCGTAGCTTAACCGGTAGCACAC
      84°C
   d. CATCGCGATCTGCAATTACGACGATAA
      GTAGCGCTAGACGTTAATGCTGCTATT
      78°C

Suppose you were to use single strands of these fragments as probes for a Southern blot.

2. If the fragments were dissolved in a solution of 50% formamide, is the stringency of hybridization higher or lower than if there were no formamide?
   Higher

3. If a high concentration of NaCl were added to the hybridization solution, how would the stringency be affected?
   The stringency would go lower.

4. Does heating of the solution from 65°C to 75°C during hybridization raise or lower stringency?
   Raise

5. At the end of the procedure, what would the autoradiogram show if the stringency was too high?
   No bands

6. In an array CGH experiment, three test samples were hybridized to three microarray chips. Each chip was spotted with 8 gene probes (genes A-H). Below are results of this assay expressed as the ratio of test DNA to reference DNA. Are any of the eight genes consistently deleted or amplified in the test samples? If so, which ones?

Gene	Sample 1	Sample 2	Sample 3
A	*1.06*	*0.99*	*1.01*
B	*0.45*	*0.55*	*0.43*
C	*1.01*	*1.05*	*1.06*
D	*0.98*	*1.00*	*0.97*
E	*1.55*	*1.47*	*1.62*
F	*0.98*	*1.06*	*1.01*
G	*1.00*	*0.99*	*0.99*
H	*1.08*	*1.09*	*0.90*

Gene B is deleted in all three samples. Gene E is amplified in all three samples.

CHAPTER 7

1. The final concentration of *Taq* polymerase is to be 0.01 unit/μL in a 50-μL PCR reaction. If the enzyme is supplied as 5 units/μl, how much enzyme would you add to the reaction?
   b. 1 μl of a 1:10 dilution of Taq

2. Primer dimers result from:
   d. 3′ complementarity in the primer sequences

3. Which control is run to detect contamination?
   d. Reagent blank

4. Nonspecific extra PCR products can result from:
   a. Mispriming

5. Using which of the following is an appropriate way to avoid PCR contamination?
   c. A separate area for PCR reaction setup

6. How many copies of a target are made after 30 cycles of PCR?
   b. 2^{30}

7. What are the three steps of a standard PCR cycle?
   Denaturation, annealing, extension

8. Which of the following is a method for purifying PCR product?
   c. Put the reaction mix through a spin column.

9. In contrast to standard PCR, real-time PCR is:
   a. Quantitative

10. In real-time PCR, fluorescence is *not* generated by
    d. *Tth* polymerase

11. Complete the following table comparing various methods of nucleic amplification:

	PCR	LCR	bDNA	TMA	Qβ Replicase	Hybrid Capture
Type of amplification	***Target***	***Probe***	***Signal***	***Target***	***Probe***	***Signal***
Target nucleic acid	***DNA***	***DNA***	***DNA or RNA***	***RNA***	***DNA or RNA***	***DNA***
Type of amplicon	***DNA***	***DNA***	***Chemiluminescent signal***	***RNA***	***RNA***	***Chemiluminescent signal***
Major enzyme(s)	***DNA polymerase***	***Ligase***	***Alkaline phosphatase***	***RNA polymerase***	***Qβ replicase***	***Alkaline phosphatase***

12. Examine the following sequence. You are devising a test to detect a mutation at the underlined position.
5′ TATTTAGTTA TGGCCTATAC ACTATTTGTG AGCAAAGGTG ATCGTTTTCT GTTTGAGATT TTTATCTCTT GATTCTTCAA AAGCATTCTG AGAAGGTGAG ATAAGCCCTG AGTCTCAGCT ACCTAAGAAA AACCTGGATG TCACTGGCCA CTGA*GGAGCT TTGTTTCAAC CAAG*TCATGT GCATTTCCAC GTCAACAGAA TTGTTTATTG TGACAGTT**A**T ATCTGTTGTC CCTTTGACCT TGTTTCTTGA AGGTTTCCTC GTCCC*TGGGC AATTCCGCAT TTAAT*TCATG GTATTCAGGA TTACATGCAT GTTTGGTTAA ACCCATGAGA TTCATTCAGT TAAAAATCCA GATGGCGAAT 3′

Design one set of primers (forward and reverse) to generate an amplicon containing the underlined base.

The primers should be 20 bases long.
The amplicon must be 100-150 bp in size.
The primers must have similar melting temperatures (Tm), +/-2°C.
The primers should have no homology in the last three 3′ bases.
Write the primer sequences 5′ → 3′ as you would if you were to order them from the DNA synthesis facility.
There are multiple answers to this question. Here is one example:
Forward: 5′ *GGAGCTTTGTTTCAACCAAG* 3′
Reverse: 5′ *ATTAAATGCGGAATTGCCCA* 3′
The product is 131 bp long. (The primer binding sites are italicized in the sequence.)

b. Write the Tm for each primer that you have designed.
Forward, 58°C Reverse, 56°C
Tm = (4°C × GC) + (2°C × AT)

CHAPTER 8

1. During interphase FISH analysis of a normal specimen for the t(9;22) translocation, one nucleus was observed with two normal signals (one red for chromosome 22 and one green for chromosome 9) and one composite red/green signal. Five hundred other nuclei were normal. What is one explanation for this observation?
Two normal chromosomes happened to fall on top of one another, resulting in an artificial "composite" signal. A low occurrence of this is accepted as background in interphase FISH.

2. Is 47; XYY a normal karyotype?
No. 46, XY is normal. This is XYY Syndrome.

3. What are the genetic abnormalities of the following genotypes?
47,XY, +18
Trisomy 18 (Edwards' syndrome)
46,XY, del(16)p(14)
Deletion in region 1, band 4 of the short arm of chromosome 16
iso(X)(q10)
Isochromosome comprised of the long arms of the X chromosome
46,XX, del(22)q(11.2)
Deletion in region 1, band 1, sub-band 2 of the long arm of chromosome 22 (diGeorge's syndrome)
45,X
Monosomy X (Turner's syndrome)

4. A chromosome with a centromere not located in the middle of the chromosome, but not completely at the end, where one arm of the chromosome is longer than the other arm, is called:
b. Acrocentric

5. A small portion of chromosome 2 has been found on the end of chromosome 15, and a small portion of chromosome 15 has been found on the end of chromosome 2. This mutation is called a(n):
a. Reciprocal translocation

6. Phytohemagglutinin is added to a cell culture when preparing cells for karyotyping. The function of the phytohemagglutinin is to:
d. Stimulate mitosis in the cells

7. A CEP probe is used to visualize chromosome 21. Three fluorescent signals are observed in the patient's cells when they are stained with this probe. These results are interpreted as consistent with:
b. Down's syndrome

8. Cells were harvested from a patient's blood, cultured to obtain chromosomes in metaphase, fixed onto a

slide, treated with trypsin, and stained with Giemsa. The resulting banding pattern is called:
a. G banding

CHAPTER 9

1. Name three assays by which the Factor V Leiden R506Q mutation can be detected.
Sequence specific PCR
PCR-RFLP
Invader™ Assay

2. Exon 4 of the HFE gene from a patient suspected to have hereditary hemachromatosis was amplified by PCR. The G to A mutation, frequently found in hemachromatosis, creates a single *Rsa*1 site in exon 4. When the PCR products are digested with *Rsa*1, what results (how many bands) would you expect to see if the patient has the mutation?
two bands

3. Which of the following methods would be practical to use to screen a large gene for mutations?
SSCP, DGGE

4. What is the phenotypic consequence of changing a codon sequence from TCT to TCC?
None. This is a silent mutation.

5. A reference sequence, ATGCCCTCTGGC, is mutated in malignant cells. The following mutations in this sequence have been described. Express these mutations using the accepted nomenclature:
ATGCGCTCTGGC
5C>G
ATGCCCTCGC
9_10delTG or 9_10del
ATAGCCCTCTGGC
2_3insA
ATGTCTCCCGGC
4_9inv
ATGATATTCTGGC
4_6delinsATAT or 4_6delCCCinsATAT

6. A reference peptide, MPSGCWR, is subject to inherited alterations. The following peptide sequences have been reported. Express these mutations using the accepted nomenclature:
MPSTGCWR
S3_G4insT
MPSGX
C5X
MPSGCWLVTGX
R7fs or R7LfsX5
MPSGR
C5_W6del
MPSGCWGCWR
4_6dupGCW

CHAPTER 10

1. Read, 5′ to 3′, the first 20 bases of the sequence in the gel on the right pictured in Figure 10-8.
5′GGAGAGGGTCCTGGGAGGGT3′

2. After an automated dye primer sequencing run, the electropherogram displays consecutive peaks of the following colors:
red, red, black, green, green, blue, black, red, green, black, blue, blue, blue
If the computer software displays the fluors from ddATP as green, ddCTP as blue, ddGTP as black, and ddTTP as red, what is the sequence of the region given?
5′TTGAACGTAGCCC3′

3. After an automated dye terminator sequencing run, the electropherogram displays bright (high, wide) peaks of fluorescence, obliterating some of the sequencing peaks. What is the most likely cause of this observation? How might it be corrected?
These are dye blobs. They can be removed by careful cleanup of the sequencing ladder after the sequencing reaction.

4. In a manual sequencing reaction, the DNA ladder on the polyacrylamide gel is very bright and readable at the bottom of the gel, but the larger (slower migrating) fragments higher up are very faint. What is the most likely cause of this observation? How might it be corrected?
The ddNTP concentration is too high.

5. In an analysis of the p53 gene for mutations, the following sequences were produced. For each sequence, write the expected sequence of the opposite strand that would confirm the presence of the mutations detected.
Normal:

5′TATCTGTTCACTTGTGCCCT3′
(Homozygous substitution)
5′TATCTGTTCATTTGTGCCCT3′
5′ AGGGCACAAATGAACAGATA3′
(Heterozygous substitution)
5′TATCTGT(T/G)CACTTGTGCCCT3′
5′ AGGGCACAAGTG(A/C)ACAGATA3′
(Heterozygous deletion)
5′TATCTGTT(C/A)(A/C)(C/T)T(T/G)(G/T)(T/G)(G/C)CC(C/T)(T/...3′
5′ AGGGCACAAGT(GA)A(A/C)(C/A)(A/G)(G/A)(A/T)(T/A)(A/...3′

6. A sequence, TTGCTGCGCTAAA, may be methylated at one or more of the cytosine residues: After bisulfite sequencing, the following results are obtained:
Bisulfite treated: TTGCTGTGCTAAA
Untreated: TTGCTGCGCTAAA
Write the sequences showing the methylated cytosines as C^{Me}.
TTGC^{Me}TGCGC^{Me}TAAA

7. In a pyrosequencing readout, the graph shows peaks of luminescence corresponding to the addition of the following nucleotides:
dT peak, dC peak (double height), dT peak, dA peak
What is the sequence?
TCCTA

CHAPTER 11

Interpretation for Case Study 11-1:

The recipient peak pattern had converted almost entirely to the donor peak pattern at 100 days. The percent residual recipient cells is calculated by analysis of the unshared alleles in this marker:

$$\%R = [R_{unshared}/(R_{unshared} + D_{unshared})] \times 100$$
$$= [3171/(40704 + 3171)] \times 100$$
$$= 7.2\%$$

At 1 year, no recipient peaks are detectable at the level of detection (0.5%–1%) of the instrument. The patient is, therefore, reported to have more than 99% donor cells and less than 1% recipient cells in the test specimen.

Interpretation for Case Study 11-2:

These results indicate that the two brothers are identical twins. This means that STR analysis cannot be used for monitoring engraftment after transplant. There is less chance of graft-versus-host-disease with this transplant; however, there is also no graft versus tumor effect that can improve the chance of removing all of the tumor cells.

Interpretation for Case Study 11-3:

The results show that the tissue was not of the same genetic origin as the patient. Apparently this microscopic fragment of tissue was introduced into one of the patient's sections during the fixing and embedding process.

1. Consider the following STR analysis:

Locus	Child	Mother	AF1	AF2
D3S1358	*15/***15**	*15*	*15*	*15/16*
vWA	*17/***18**	*17*	*17/18*	*18*
FGA	*23/***24**	*22/23*	*20*	*24*
TH01	*6/***10**	*6/7*	*6/7*	*9/10*
TPOX	*11/***11**	*9/11*	*9/11*	*10/11*
CSF1PO	*12/***12**	*11/12*	*11/13*	*11/12*
D5S818	*10/***12**	*10*	*11/12*	*12*
D13S317	***9**/10*	*10/11*	*10/11*	*9/11*

a. Circle the child's alleles that are inherited from the father.
Bold numbers

b. Which alleged father (AF) is the biological parent?
AF2

2. The following evidence was collected for a criminal investigation.

Locus	Victim	Evidence	Suspect
TPOX	*11/12*	*12,* **11/12**	*11*
CSF1PO	*10*	**10**, *9/10*	*9/10*
D13S317	*8/10*	*10,* **8/10**	*9/12*
D5S818	*9/11*	*10/11,* **9/11**	*11*
TH01	*6/10*	**6/10,** *8/10*	*5/11*
FGA	*20*	**20,** *20/22*	*20*
vWA	*15/17*	*18,* **15/17**	*15/18*
D3S1358	*14*	*15/17,* **14**	*11/12*

The suspect is heterozygous at the amelogenin locus.

a. Is the suspect male or female?
Male

b. In the evidence column, circle the alleles belonging to the victim.
Bold numbers

c. Should the suspect be held or released?
Released

3. A child and an alleged father (AF) share alleles with the following paternity index:

Locus	Child	AF	Paternity Index for shared allele
D5S818	*9,10*	*9*	*0.853*
D8S1179	*11*	*11,12*	*2.718*
D16S539	*13,14*	*10,14*	*1.782*

a. What is the combined paternity index from these three loci?
0.853 × 2.718 × 1.782 = 4.131

b. With 50% prior odds, what is the probability of paternity from these three loci?
(4.131 × 0.5)/[(4.131 × 0.5) + 0.5)] = 0.80 or 80%

4. Consider the following theoretical allele frequencies for the loci indicated:

Locus	Alleles	Allele Frequency
CSF1PO	*14, 14*	*0.332*
D13S317	*9, 10*	*0.210, 0.595*
TPOX	*8, 11*	*0.489, 0.237*

a. What is the overall allele frequency, using the product rule?
$0.332 \times 0.332 \times 0.21 \times 0.595 \times 0.489 \times 0.237 = 1.596 \times 10^{-3}$

b. What is the probability that this DNA found at the two sources came from the same person?
$1/1.596 \times 10^{-3} = 626.5$

5. STR at several loci were screened by capillary electrophoresis and fluorescent detection for informative peaks prior to a bone marrow transplant. The following results were observed:

Locus	Donor Alleles	Recipient Alleles
LPL	*7, 10*	*7, 9*
F13B	*8, 14*	*8*
FESFPS	*10*	*7*
F13A01	*5, 11*	*5, 11*

Which loci are informative?
F13B is donor informative. FESFPS and LPL are informative.

6. An engraftment analysis was performed by capillary gel electrophoresis and fluorescence detection. The fluorescence as measured by the instrument under the FESFPS donor peak was 28,118 units and that under the FESFPS recipient peak was 72,691. What is the percent donor in this specimen?
28118/(28118 + 72691) = 28% donor

7. The T-cell fraction from the blood sample in Question 6 was separated and measured for donor cells. Analysis of the FESFPS locus in the T-cell fraction yielded 15,362 fluorescence units under the donor peak and 97,885 under the recipient peak. What does this result predict with regard to T-cell mediated events such as graft-versus-host disease (GVHD) or graft-versus-tumor (GVT)?
15362/(15362 + 97885) = 13.6% donor cells; GVDH or GVT is unlikely

8. If a child had a Y haplotype including DYS393 allele 12, DYS439 allele 11, DYS445 allele 8, and DYS447 allele 22, what are the predicted Y alleles for these loci of the natural father?
DYS393 allele 12, DYS439 allele 11, DYS445 allele 8, and DYS447 allele 22

9. Which would be used for a surname test, Y-STR, mitochondrial typing or autosomal STR?
Y-STR

10. An ancient bone fragment was found and claimed to belong to an ancestor of a famous family. Living members of the family donated DNA for confirma-

tion of the relationship. What type of analysis would likely be used for this test? Why?
Mitochondrial DNA typing might be indicated, because (1) the small circular, naturally amplified mitochondrial DNA is more likely to be attained from the old sample and (2) lineage across several generations can be determined using the maternal inheritance of mitochondrial type.

11. What are two biological exceptions to positive identification by autosomal STR?
Identical twins and clones have identical nuclear DNA profiles.

CHAPTER 12

Interpretation for Case Study 12-1:

Norovirus cannot be cultured. Laboratory tests include electron microscopy, serology, and RT-PCR. Electron microscopy and immune electron microscopy require specialized equipment and technical expertise. Detection of serum antibodies to the virus is not always straightforward, as most of the adult (but not child) population has serum antibodies to this virus. Furthermore, it can take several days after exposure to develop detectable IgM antibodies. RT-PCR, therefore, is the method of choice for detection of this RNA virus. The gene target is the viral RNA polymerase. Using this target, a broad spectrum of noroviral types can be detected. The results indicated that the virus was present in salad lettuce served at the hotel. Lettuce sampled directly from the distributor did not carry virus, indicating that the contamination occurred at the hotel. This assumption was supported by the discovery of viral RNA in hotel employees who had prepared the food. Direct sequencing of the RT-PCR products revealed identical sequences for all positive specimens, confirming that the guests, workers, and food source shared the same viral strain.

Interpretation for Case Study 12-2:

Results from the culture of the isolates were consistent with MRSA. The results from the PFGE analysis indicated that all except one of the isolates from the students were the same strain. One isolate exhibited two differences from the others, indicating that it was closely related to these *S. aureus* isolates. Resistance to oxacillin/methicillin results from the expression of an altered penicillin-binding protein encoded by the *mecA* gene. All five isolates shared the type IV *mecA* gene, associated with MRSA. PVL, also found in these isolates, is thought to be responsible for tissue necrosis in MRSA infections.

Further investigation into the cases revealed that all the students had participated in a wrestling meet at one of the high schools. Passage of the organism during this event was the likely source of the infection. The meet location was thoroughly cleaned according to CDC recommendations, and students were encouraged to always wash their hands and maintain good hygiene.

Interpretation for Case Study 12-3:

The fact that the patient's viral loads were gradually increasing was a sign that the virus was developing resistance to the antiviral drugs. Slight variations of viral quantity within 0.3 $\log_{10}$ units are considered normal. This patient, however, was seeing significant increases in viral replication over the last 6 months.

This patient's virus has a mutation in the reverse transcriptase gene that has made the virus resistant to AZT. The patient's drug treatment needs to be changed immediately, with AZT being replaced by another reverse transcriptase inhibitor that would be unaffected by this mutation, such as didanosine or lamivudine. Viral load measurements should be taken regularly to make sure that the change in drug therapy causes a decrease in the viral load over the next few months. Genotyping should be performed again if the viral load starts to trend up.

1. Which of the following genes would be analyzed to determine whether an isolate of *Staphylococcus aureus* is resistant to oxacillin?
a. *mecA*

2. Which of the following is a genotypic method used to compare two isolates in an epidemiological investigation?
c. Ribotyping

3. For which of the following organisms does caution need to be exercised when evaluating positive PCR results because the organism can be found as normal flora in some patient populations?
b. *Streptococcus pneumoniae*

4. Which of the following controls are critical for ensuring that amplification is occurring in a patient sample

and that the lack of PCR product is not due to the presence of inhibitors?
d. Amplification control

5. A PCR assay performed to detect *Bordetella pertussis* on sputum obtained from a 14-year-old girl who has had a chronic cough had two bands, one consistent with the internal control and the other consistent with the size expected for amplification of the *B. pertussis* target. How should these results be interpreted?
b. The girl has clinically-significant *B. pertussis* infection

6. Which of the following is a disadvantage of molecular-based testing?
a. Results stay positive longer after treatment than do cultures

7. A molecular-based typing method that has high typing capacity, reproducibility, and discriminatory power, moderate ease of performance, and good to moderate ease of interpretation is:
c. PCR-RFLP

8. A patient has antibodies against HCV and a viral load of 100,000 copies/mL. What is the next test that should be performed on this patient's isolate?
d. Inno-LiPA HCV genotyping

CHAPTER 13

1. Which of the following is not a triplet repeat expansion disorder?
c. Factor V Leiden

2. A gene was mapped to region 3, band 1, sub-band 1 of the long arm of chromosome 2. How would you express this location from an idiogram?
2q31.1

3. Which of the following can be detected by PCR?
c. Mitochondrial point mutations

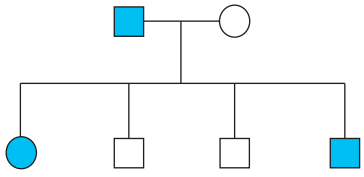
4. A patient was tested for Huntington's disease. PCR followed by PAGE revealed 25 CAG units.
c. This patient is normal at the Huntingtin locus.

5. The factor V Leiden mutation can be detected by:
d. All of the above methods

6. The most frequently occurring mutation in the *HFE* gene results in the replacement of cysteine (C) with tyrosine (Y) at position 282. How is this expressed according to the recommended nomenclature?
C282Y

7. MELAS is a disease condition that results from an A to G mutation at position 3243 of the mitochondrial genome. This change creates a single *Apa*I restriction site in a PCR product, including the mutation site. What would you expect from a PCR-RFLP analysis for this mutation on a patient with MELAS?
b. A single PCR product that cuts into two fragments upon digestion with *Apa*I

8. A father affected with a single gene disorder and an unaffected mother have four children (three boys and a girl), two of whom (one boy and the girl) are affected. Draw the pedigree diagram for this family.



D16S539, an STR, was analyzed in the family. The results showed that the father had the 6,8 alleles, and the mother had the 5,7 alleles. The affected children had the 5,6 and 6, 7 alleles, and the unaffected children had the 5,8 and 7,8 alleles.
a. If D16S539 is located on chromosome 16, where is the gene for this disorder likely to be located?
Chromosome 16
b. To which allele of D16S539 is the gene linked?
The 6 allele
How might a DNA analysis be performed for the presence of the disorder?
a. Analyze D16S539 for the 6 allele by PCR.

9. Exon 4 of the HFE gene from a patient suspected to have hereditary hemachromatosis was amplified by

PCR. The G to A mutation, frequently found in hemachromatosis, creates an *Rsa*1 site in exon 4. When the PCR products are digested with *Rsa*1, which of the following results would you expect to see if the patient has the mutation?

c. The patient's PCR product will yield extra bands upon *Rsa*1 digestion.

10. Most people with the C282Y or H63D *HFE* gene mutations develop hemachromatosis symptoms. This is because of:

c. High penetrance

CHAPTER 14

1. What are the two important checkpoints in the cell division cycle that are crossed when the regulation of the cell division cycle is affected?

G1 to S and G2 to M

2. An EWS-FLI-1 mutation was detected in a solid tumor by RT-PCR. Which of the following does this result support?

b. Ewing's sarcoma

3. Mutation detection, even by sequencing, is not definitive with a negative result. Why?

Mutations may exist outside the sequenced area.

4. A PCR test for the bcl-2 translocation is performed on a patient with suspected follicular lymphoma. The results show a bright band at about 300 bp for this patient. How would you interpret these results?

If 300 bp is within the expected size limits for translocation breakpoints, the patient is positive for the t(14;18) or bcl-2 translocation.

5. Which of the following misinterpretations would result from PCR contamination?

a. False positive for the t(15;17) translocation

6. After amplification of the t(12;21) breakpoint by RT-PCR, the PCR products along with the proper molecular weight standard were loaded and resolved on an agarose gel. What might be the explanation for each of the following observations when the gel is exposed to UV light? (Assume that positive and amplification controls and a reagent blank control are included in the run.)

a. The gel is blank (no bands, no molecular weight standard).
Ethidium bromide or Sybr green staining was omitted.

b. Only the molecular weight standard is visible.
Reverse transcriptase or PCR reaction did not work.

c. The molecular weight standard is visible. There are bands in every lane at 200 bp, even in the reagent blank lane.
PCR contamination is present.

7. What is observed on a Southern blot for gene rearrangement in the case of a *positive* result?

b. Germline bands plus rearranged bands

8. Cyclin D1 promotes passage of cells through the G1 to S checkpoint. What test detects translocation of this gene to chromosome 14?

c. t(11;14) translocation analysis *(BCL1/IGH)*

9. Why is the Southern blot procedure superior to the PCR procedure for detecting clonality in some cases?

b. The PCR procedure cannot detect certain gene rearrangements that are detectable by Southern blot.

10. Interpret the following results from a translocation assay.
Are the samples positive, negative, or indeterminate?

Sample 1: Negative
Sample 2: Indeterminate, did not amplify
Sample 3: Positive

CHAPTER 15

Interpretation for Case Study 15-1:

This patient most likely has celiac disease. Although the presence of these alleles cannot always be diagnostic of the disease, their absence excludes celiac disease as a likely diagnosis. The heterozygous DQB1 locus is associated with less severe symptoms than if the *0201 allele

is homozygous. A more comprehensive test list would include detection of total IgA antibodies, autoantibodies, reticulin and transglutaminase, and gliadin IgG and IgA antibodies. The SBT test was useful in this case because the serological results were not clear.

Interpretation for Case Study 15-2:

Donor 3 was selected as compatible, with two mismatches, one at HLA-A and one at HLA-B. These mismatches are in the graft-versus-host direction; that is, the graft would recognize the host as foreign more effectively than the host immune system will recognize the graft. After the transplant, the patient remained in a state of split chimerism for a year. At that time, 71% of monocytes, 62% of granulocytes, and 99% of lymphocytes were donor-derived. Throughout this time, the tumor gene rearrangement was not detectable by PCR, and the patient remained in remission more than a year post transplant.

Interpretation for Case Study 15-3:

The alleles detected by SSP-PCR are identified using a worksheet that converts the lane numbers to allele specificity. Using this worksheet, primers in lanes 7 and 19 amplify the A*2419, and those in lane 22 amplify the A*3401 allele. The donor organ is a good match for this recipient. Mother and daughter are expected to match at least half of the HLA alleles. The presence of serum antigens was determined to predict the risk of rejection. Although antihuman antibodies were detected using flow cytometry and bead array technology, crossmatching results indicated that the antibodies were a consequence of the lupus and were not directed at the donor antigens. Even though the patient has lupus, the risk of rejection is low as established by the antigen and antibody studies. Following the transplant, the recipient will have to take immunosuppressive medication. Follow-up serum antibody tests may be performed to monitor the state of the transplant.

1. Which of the following is a high-resolution HLA typing result?
   c. A*0212

2. Which of the following is a likely haplotype from parents with A25,Cw10,B27/A23,Cw5,B27 and A17, Cw4,B10/A9,Cw7,B12 haplotypes?
   a. A25,Cw10, B27

3. Upon microscopic examination, over 90% of cells are translucent after a CDC assay. How are these results scored according to the ASHI rules?
   90%–100% viability (unstained cells) is scored as 1 or negative.

4. An HLA-A allele is a CTC to CTT (leu → leu) change at the DNA level. How is this allele written?
   b. HLA-A*0201

5. A candidate for kidney transplant has a PRA of 75%. How will this affect eligibility for immediate transplant?
   Because this patient has antibodies likely to react to 75% of the population, eligibility will be negatively affected.

6. An SSOP probe recognizes HLA-DRB*0301-0304. Another probe recognizes HLA-DRB*0301/0304, and a third probe hybridizes to HLA-DRB*0301-0303. Test specimen DNA hybridizes to all except the third probe in a reverse dot blot format. What is the HLA-DRB type of the specimen?
   The HLA type is HLA-DRB*0304

7. What is the relationship between alleles HLA-A10 and HLA-A26(10)?
   HLA-A10 is the parent allele of HLA-A26(10)

8. A CDC assay yields an 8 score for sera with the following specificities: A2, A28 and A2, A28, B7, and a 1 score for serum with an A2 specificity. What is the HLA-A type?
   The HLA-A type is A28

9. HLA-DRB1*1501 differs from DRB1*0101 by a G to C base change. If the sequence surrounding the base change is: …GGGTGCGGTTGCTGGAAA GAT… (DRB1*0101) or …GGGTGCGGTTCCTG GAAAGAT… (DRB1*1501), which of the following would be the 3′ end of a sequence-specific primer for detection of DRB1*1501?
   d. …ATCTTTCCAGG

10. The results of an SSP-PCR reaction are the following: lane 1, one band; lane 2, two bands, lane 3 no bands. If the test includes an amplification control

multiplexed with the allele-specific primers, what is the interpretation for each lane?
The test specimen does not contain the allele recognized by the primers in lane 1. Only the amplification control is visible.
The test specimen contains the allele recognized by the primers in lane 2. Both the amplification control and the allele-specific product are visible.
The PCR reaction did not work for the reaction run in lane 3.

CHAPTER 16

What actions should be taken in the following situations?

1. An unlabeled collection tube with a requisition for a factor V Leiden test is received in the laboratory.
   Notify the supervisor and reject the specimen.

2. After PCR, the amplification control has failed to yield a product.
   Check the original DNA or RNA preparation. If it is adequate, repeat the amplification. If not, reisolate the nucleic acid.

3. An isolated DNA sample is to be stored for at least 6 months.
   Store at -70°C in a tightly sealed tube.

4. A bone marrow specimen arrives at the end of a shift and will not be processed for the Bcl2 translocation until the next day.
   Place the specimen in the refrigerator.

5. The temperature of a refrigerator set at 8°C (±2°C) reads 14°C.
   Recheck the temperature after a few hours. If it does not return to range, notify the supervisor.

6. A PCR test for the BCR/ABL translocation was negative for the patient sample and for the sensitivity control.
   Repeat the PCR with the addition of a new sensitivity control.

7. A fragile X test result has been properly reviewed and reported.
   File the test results, documents, and associated autoradiographs together in the laboratory archives.

8. A bottle of reagent alcohol with a 3 in the red diamond on its label is to be stored.
   Place the alcohol bottle in a safety storage cabinet for flammable liquids.

9. The expiration date on a reagent has passed.
   Discard the reagent. If it can be used for research or other nonclinical purposes, label and store it in a separate area away from patient testing reagents.

10. Test results are to be faxed to the ordering physician.
    Fax the results with a cover sheet containing the proper disclaimer.

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