IN SITU PCR

TECHNIQUES

OMAR BAGASRA
JOHN HANSEN

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OMAR BAGASRA, M.D., Ph.D.
Molecular Retrovirology Laboratories
Thomas Jefferson University
Jefferson Medical College
Philadelphia, Pennsylvania

JOHN HANSEN
MJ Research, Inc.
Watertown, Massachusetts

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FOREWORD

Mullis's invention of the polymerase chain reaction (PCR) and its application to the detection and analysis of specific nucleic acid sequences revolutionized virtually all areas of molecular biology and has given us a wealth of new applications. Similarly, perfection of techniques for gene amplification in situ may be expected to fuel accelerated developments in the heightened understanding of embryogenesis and organogenesis as well as the pathogenesis of infectious, genetic, immunologic, neoplastic, and other disease processes. More than 300 papers describing the use of these techniques have already appeared, but the procedures remain cumbersome and difficult to reproduce, with many potential variables (as reviewed by Komminoth and Long). Bagasra and co-workers have provided a great service in giving us this comprehensive protocol. They have included hints and laboratory secrets that frequently get left out of scientific papers, making repetition of published experiments so problematic. This monograph should prove immensely useful not only for the novice but also for the experienced worker striving for maximum specificity or sensitivity in qualitative searches for cells containing low levels of genes or messenger as well as for those seeking greater sensitivity, precision, and reproducibility in enumeration of such cells.

As one of the true pioneers in the field, Bagasra has brought a wealth of experience to the preparation of this protocol. I recall our first discus-

sion of in situ amplification in his laboratory at the University of Medicine and Dentistry of New Jersey. We were preparing a collaborative protocol for study of the transmission of HIV-1 from mother to fetus using conventional PCR. In the course of the discussion, we realized that although PCR had great potential for early diagnosis of infant infection for studies of pathogenesis it would be incalculably valuable to be able to use the same amplification technique to detect individual HIV-1-infected cells, to distinguish between latently and productively infected cells, and to differentiate maternal from infant cells in tissue sections and in cytocentrifuged or cytofluorographed cell suspensions. I guessed that a number of large laboratories would be working on such applications and urged Bagasra—at the time a lone researcher—not to enter the race. Fortunately, he did not heed my advice and, in fact, spent much of his spare time developing his technique for in situ amplification. By the time he moved to Temple University and St. Christopher's Hospital for Children in 1990, he had already found at least preliminary solutions to the most vexing problems, and he made the system work for complementary HIV-1 DNA in presumed latently infected cells in which HIV-1 DNA and RNA were undetectable by direct hybridization. This was described in the Perkin Elmer Cetus periodical *Amplifications*. He and I filed half a dozen grant applications to develop the technique or use it in conjunction with other assays for study of the pathogenesis of perinatal AIDS infection—none of which was funded. Rather, most were returned with bitter criticism by experts, who claimed that it would be impossible to preserve cell integrity at the temperatures required to anneal DNA and impossible to prevent diffusion of the new amplification products from the cell—even after data and photomicrographs were presented to show that these problems had already been partially surmounted. Because we were unable to fund his work, by 1991 Bagasra had to move to Thomas Jefferson University. At that time, the assay had become sufficiently precise in recovery of HIV-1-infected cells added to populations of uninfected cells and reproducible to such a degree as to give us confidence in our findings that both adults and children had many more infected cells in their peripheral blood than had previously been suspected. At Jefferson, Bagasra refined the assay; definitively documented the relatively high proportion of CD4+ lymphocytes and monocytes containing HIV-1 DNA; and went on to initiate innovative applications of the technique to a variety of basic problems, particularly the pathogenesis of HIV-1 infection.

In 1990, Haase and co-workers, working independently of Bagasra, reported success in accomplishing in situ amplification with an interesting innovation: the use of a set of multiple overlapping primers to produce amplification products spanning more than 1000 base pairs, which are large enough to slow diffusion from their site of origin. However, the efficiency of amplification in this system is low, and Bagasra found that the large number of cycles required to get sufficient amplification frequently leads to more nonspecific labeling than with the use of single primer sets. Nuovo and co-workers implemented yet another innovation, that of incorporating digoxigenin-labeled nucleotides into the PCR amplicons so that the in situ PCR products might be detected directly by histochemical techniques. Komminoth and co-workers, however, have shown that such direct labeling is frequently associated with a false-positive labeling of cells. Patterson and Wolinsky developed a curious variation: amplifying and labeling cellular signals in tubes, then characterizing the cells through flow cytometry. Bagasra's approach to in situ amplification of gene sequences (described in this protocol) appears to be especially sensitive and specific. Specificity can be further increased by use of more than one set of primers for different genes from the same organism or from different regions of the same gene or message.

Even with the aid of this manual, today gene amplification in situ remains a difficult research procedure. Conceptually, however, it offers tremendous potential as an aid to clinical diagnosis through its ability to detect a single copy of a specific microbial, neoplastic, messenger, or mutated nucleic acid sequence in a cell smear, cell suspension, tissue section, or chromosome. A key challenge for the future will be the simplification of these techniques so they can be brought to the laboratory of the clinical pathologist.

HAROLD W. LISCHNER, M.D.

Professor of Pediatrics and Microbiology/Immunology
Chief of Pediatric Immunology Section
Director of Pediatric AIDS Program
Temple University School of Medicine and
St. Christopher's Hospital for Children
Philadelphia, Pennsylvania

PREFACE

I know nothing except the fact of my ignorance.
—Socrates, as reported by Diogenes Laetius

Possibly we shall one day know a little more than we do now.
But the real nature of things, that we shall never know, never.
—Albert Einstein

Pass beyond forms, escape from names, flee titles and awards, walk towards meaning.
—Rumi

In situ polymerase chain reaction (PCR) is a powerful invention, allowing an investigator to visualize the presence of a single-copy gene, an individual virus, or a low abundance RNA signal in its original location inside a cell or tissue. Never before has a technique quite so sensitive been available to scientists who work in cytology or histology, and it is our hope that reproducible practice of the technique will serve as a wellspring from which will flow new understandings in biology and medicine.

Simultaneously, we wish to point out that in situ PCR is a relatively new technique that still exhibits characteristics of both science *and* art. DNA and RNA are, by themselves, each exceedingly complex phenom-

ena; but when these genetic molecules do their thing inside the complex milieu of whole cells and tissues, they sometimes exhibit characteristics that will probably always defy complete understanding. Empirical work has led to substantial knowledge of what works and what does not experimentally; and in this volume, we communicate this practical knowledge as best we can. But readers should realize that in situ PCR represents a sharp edge in research; sometimes it cuts through to greater understanding and sometimes it just cuts the self-confidence of those investigators who choose to play with it.

By no means do we wish to discourage any scientist from trying in situ PCR; rather we just wish to point out that ignorance still abounds in the field, and many promising pathways remain unexplored. This represents great opportunity for the imaginative scientist, and we encourage investigators to explore what might seem to be, at first glance, far-fetched ideas. But please practice a bit with simpler systems first, for much practical knowledge on the manipulation of signals, cells, and detection systems must be learned before proceeding onto the pioneering work.

Where has this protocol come from? For the past four years, one of us (OB) has offered numerous workshops and seminars on in situ PCR in an effort to communicate to as many investigators as possible the in situ techniques that were developed in his laboratory at Thomas Jefferson University. Simultaneously, the other author (JH) has worked in industry to develop instrumentation to drive the reaction with increasing ease and reproducibility. Between the two of us, we have listened to and tried to answer the myriad questions that constantly arise from the ever-curious seminar participants and from inquiring scientific colleagues. In doing so, we have learned much about what matters need special clarification, where technical problems often arise, and how to best present the subject matter in a manner that will be comprehensible to nonspecialists in molecular biology as well as to nonspecialists in tissue morphology.

We have tried to cover as many aspects of in situ PCR as possible, but we have included only those aspects with which we have personal experience. Thus this volume represents a specific viewpoint, and it is not intended to be a comprehensive textbook of all matters pertaining to in situ PCR. Rather it is intended to be a practical manual for use in the laboratory, and we hope the many photos will prove especially instructive.

We are especially indebted to Sashamma Thikavetapu, Lisa Bobroski, and Patricia Whittle, who assisted in numerous workshops and who gave us many excellent suggestions. We also wish to thank the entire staff of

the in situ laboratory at Thomas Jefferson University, all of whom have played an important role in the development of this protocol. We also wish to thank the Bagasra family—Theresa, Alexander, and Anisah—who cheerfully tolerated the two of us trying to iron out the various details while working in the basement of the Bagasra home in Laurel Springs.

Last, we wish to thank the many scientific colleagues who participated in the in situ seminars, for they provided the impetus for the development of this protocol. We have always been most impressed by the imagination of these individuals, who are constantly proposing new and exciting applications for in situ PCR. It is our fervent hope that you, the reader, will prove to be just as imaginative. In fact, it is our fervent hope that this very volume will become stained with the many colors that can now be used for multiplexed hybridization of PCR products—marks that you leave behind as you blaze new pathways to scientific discovery.

CHAPTER 1

REVIEW OF THE PCR TECHNIQUE

This chapter is a primer for those investigators for whom molecular techniques are a relatively new experience. Many of those interested in performing in situ polymerase chain reaction (PCR) do not come from a molecular biology background; rather they are often pathologists, for example, skilled in careful tissue preparation and exacting histological analysis but not in the design of oligonucleotide primers and probes. Thus this chapter is an overview of the relevant molecular information. It reviews the history of the PCR technique, describes how it works, and details many of the technical points important for successful gene amplification. For those already familiar with these matters, perhaps the sections on the design of primers and probes might prove useful, particularly in regard to obtaining sequence information over the Internet. Otherwise, those investigators skilled in molecular biology might wish to skip to subsequent chapters, where the in situ methodologies are detailed.

KARY MULLIS'S INVENTION: THE POLYMERASE CHAIN REACTION

In the mid-1980s, Kary Mullis, a biochemist working for the Cetus Corporation, invented a method to identify a specific DNA sequence in an

aqueous solution that contains myriad sequences of DNA and then to geometrically amplify the targeted sequence millionsfold through a semi-automated procedure that takes just 1 or 2 h. This synthetic process makes available enough of the targeted DNA for ready analysis by conventional laboratory techniques, even if there was just one molecule of the DNA in the solution to start with.

According to Mullis, this invention originally sprang from a scheme he was devising to keep employed several technicians who worked in his laboratory. The lab specialized in synthesizing short chains of DNA called oligonucleotide primers (oligos), and apparently demand within the Cetus Corporation for these synthesized molecules was insufficient to justify the level of staffing Mullis was then enjoying. He was, therefore, trying to devise some sort of new application for the oligos that would consume large quantities of the lab's product. Following his famous drive one weekend up the Mendicino coast of California and his "Eureka!" experience at a highway rest area, the polymerase chain reaction (PCR) was born.

Since those salad days of PCR, methods have been found to simplify the procedure, particularly through the use of thermally stable polymerase enzyme, and to automate the process, through the adaptation of microprocessor-controlled thermal cyclers. Furthermore, reverse transcriptase (RT) reactions have been added, if one wishes to identify and amplify targeted RNA sequences by first converting them to cDNA templates; this is sometimes called RNA PCR.

Application of PCR has now spread far and wide throughout biotechnology. In particular, the reaction has found many uses in molecular biology laboratories for identifying and generating large quantities of DNA for routine assays, for example, as well as for cloning, gene mapping, and engineering new forms of DNA through in vitro mutagenesis. In medicine, PCR has proven useful not only for the identification of infectious agents but also in the diagnosis of genetic disease and in the understanding of the pathogenesis of disease processes. In forensic science, PCR has revolutionized the practice of DNA fingerprinting and HLA typing. In plant genetics, PCR has created new tools for analyzing and accelerating breeding experiments as well as easing the chore of precise taxonomic classification. The list goes on and on; and in 1993, the Nobel Prize Committee recognized Mullis's seminal contribution and awarded him the Nobel Prize in Chemistry for his invention.

THE MECHANISM OF THE CHAIN REACTION

How exactly does PCR work? Actually, the reaction represents one of those amazingly simple insights that in hindsight almost every investigator wonders why he or she did not think of it first. The researcher simply takes a sample of biological material that contains DNA (or RNA) and processes it to extract the nucleic acids; this extraction procedure can often be as simple as boiling the specimen for 10 min with chelex resin. The biological sample can be almost anything: a bacterial culture, the root of a hair follicle, the pulp of an ancient tooth, a tissue sample from an autopsy performed thirty years ago, or even a fossil leaf from a peat bog. After the sample of DNA is free of its matrix, it is placed into a small tube along with an aliquot of a thermally stable DNA polymerase enzyme (a protein that constructs DNA from components); some salts which are necessary for polymerase to function; and a supply of the particular nucleotides from which DNA is built, called deoxyribonucleoside triphosphates (dNTPs). Last, a set of critical ingredients is added—the aforementioned oligonucleotide primers, which are short segments of single-stranded DNA that have been artificially produced in a DNA synthesizer. These primers initiate the chain reaction.

Oligonucleotide Primers

Primers are typically 18 to 22 bases long, and they represent the exact DNA sequences at the beginning and at the end of the gene of interest. The reason they are only 18 to 22 bases long is that there are four possible nucleotides at any specific spot in DNA (ie, G, A, T, or C), so each locus has one chance in four of being any particular one. The chance of a random match for a 20-mer primer (or 20–base pair oligo primer) to any anonymous strand of DNA would be $1:4^{20}$, or less than one chance in a trillion.

These primers are, therefore, extremely specific to the precise genetic sequence an investigator is seeking. But how is a researcher to know exactly what the primer sequence should be? Usually you rely on knowledge that has been previously gleaned about particular genes, and there is an enormous databank—called GenBank, accessed through the Los Alamos National Laboratory and the National Library of Medicine—that supplies such information through the World Wide Web or via modem

free of charge (**http://www.ncbi.nlm.nih.gov**). A researcher can also look to the scientific literature for published sequences from prior experiments or can purchase preoptimized primers from a vendor.

However, for the genetic pioneers, knowledge of the DNA sequence must often be developed de novo, perhaps by reverse transcribing and sequencing an mRNA of interest or perhaps by reading a protein then figuring out all the possible DNA sequences that could have encoded it. Then the investigator prospects the genome with something known as *degenerate primers,* which are mixed sets of primers that can accommodate all the possible sequences that can encode the protein of interest. When trying to do something new, designing primers can take a lot of thought and effort.

Denaturation and Annealing

After the primers have been made, they are put into a plastic tube with the other reaction components, and the tube is sealed and placed into a thermal cycler. The cycler then drives the mixture up to denaturation temperature, typically 92° to 94°C. At these temperatures, the hydrogen bonds between the base pairs along the two strands of the double helix of DNA become so strained that they can no longer hold the two strands together. The DNA molecules in the specimen separate (or denature), and each molecule becomes two completely independent, but complementary, single-stranded molecules of DNA.

Then the thermal cycler drives the temperature of the mixture down to a predetermined annealing temperature, usually between 50° and 60°C. At these temperatures, the various single strands of DNA are urgently looking for complementary mates with whom they can pair bond, if the two match up exactly. However, the original mates of the long strands of experimental DNA find themselves vastly outnumbered by all the smaller bits of DNA—the synthetic oligo primers. The relatively high concentration of the primers (usually more than 10^{10} to 10^{11} per reaction) makes them ubiquitous in the solution, at least compared to the relatively few copies of the original strands of DNA. If a primer molecule should discover that one of the long strands of DNA contains a complementary region that exactly matches its 18 to 22 bases, the primer latches onto the long strand almost instantly (or anneals). Together, the primer and the long strand form a short segment of double-stranded DNA, with the remaining part of the long molecule flopping about as a single strand.

Extension

Meanwhile, molecules of DNA polymerase protein are floating around, looking for work like unemployed construction engineers. The work order the polymerase molecules are seeking is a single strand of DNA dangling from a double-stranded segment, where the end of the double segment has a blunt 3′ end and the remaining single strand is dangling. DNA polymerase enzyme will hop right on this situation, namely onto a long strand of parental DNA with an annealed primer, and it reads the bases, one at a time, along the parental strand. The polymerase enzyme grabs out of solution the complementary nucleotide from all the dNTPs floating around, and jams it into proper position at the 3′ end of the primer, to form a new bonded base pair between the two strands. The polymerase molecule continues to do this time and time again, copying the parental strand and extending the primer so that the primer itself eventually becomes a freshly synthesized long strand of complementary DNA.

All this occurs in a matter of seconds after the tube and reaction mixture arrive at the annealing temperature. However, so much is going on at this lower temperature—every single-stranded DNA is desperately seeking a complementary mate—that experience has proven that raising the incubation temperature to an *extension* temperature around 72°C for a while helps improve fidelity among the single strands of DNA. The polymerase enzyme works just fine at this higher temperature (in fact, it is the optimal work environment for *taq* polymerase), and the enzyme has time to complete its chore of extending the primer. However, at this elevated temperature, single strands of DNA are now more content to remain single, and they have less tendency to mess around with the wrong sort. (At lower temperatures, mismatches can occur between primers and other single-stranded DNA molecules when the sequences are only partly complementary but sufficiently so that primer and DNA will anneal long enough for the polymerase enzyme to hop on and extend the primer and create a new strand. This false priming can result in nonspecific amplification and a consequent decrease in the specificity of the reaction.)

Second Thermal Cycle

After extension is complete, the thermal cycler drives the reaction cocktail back up to denaturation temperature. All the double-strands of DNA,

including the newly synthesized ones, melt into two single strands. Then the thermal cycler drives the mixture back down to annealing temperature, and once again every single-stranded DNA molecule starts shopping around for a perfect mate.

Again, the primers have the edge, because they are so numerous; but this time the situation is slightly different. Remember there were two types of primers, one from the beginning and one from the end of the sequence. Well their design is rather clever, because they match the beginning and end of the targeted gene sequence—but on the opposite, complementary, strands. So in the first cycle of annealing and extension, both primers were extended, but one extended from the *beginning* of the gene on one of the parental strands of DNA, and the other primer extended backward from the *end* of the gene on the complementary parental strand. Thus the first cycle resulted in twice as many copies of the gene than there were in the beginning. But the first-generation copy of a parental strand is always of indeterminate length, because the polymerase enzyme extends as long as it can; it just does not know where to stop.

However, this first copy has a very specific terminus at the 5′ end of the DNA molecule, which is the 5′ end of the original primer that got the whole thing going in the first place. Now that the annealing process is occurring once again, the *other* type of primer looks for its complementary region on the first-copy long strand. When the primer finds this sequence, it anneals to the long strand and waves it 3′ end, signaling polymerase enzyme to jump on. Once again, the polymerase enzyme extends the primer, this time in the other direction, reading from the longer strand and attaching the appropriate dNTPs. But when it reaches the 5′ end of the long strand, the end of the original primer, it falls off to seek work elsewhere. Thus the second-generation copy has a specific length; it is, in fact, the *exact gene* that the researcher designed the primers to seek out and amplify.

Geometric Amplification

This cycle of denaturation–annealing–extension is repeated again and again, usually about 30 times, ultimately becoming a chain reaction of DNA synthesis. There is a doubling of the gene of interest with each cycle, as copies are made of copies, resulting in tens or perhaps hundreds of millions of copies of the desired gene at the end of the procedure, even if there was as few as *one* copy of the gene at the start. Furthermore, it is the nature of the mathematics that the first copies (the long copies of in-

determinate length) accumulate only arithmetically, while the second and subsequent generations of copy (the ones of particular length) amplify geometrically. Therefore, virtually all the copies at the end of the run are the desired gene *and* of a specific length.

And what happens if the gene of interest for which the primers were designed was never in the original sample of DNA that got put into the tube? Why, there is no annealing of the primers, no attachment of polymerase, no first-generation copies, no subsequent copies—no amplification whatsoever. Totally negative results, none, caput, no lanes in the gel; you end with what you started with.

Of course, real world complexities enter into the process: the temperatures may not be just right, the salts are not to taste, the primers do not quite anneal properly. For these reasons, the procedure rarely goes to theoretical perfection; but the reaction is, in fact, remarkably robust and quite predictable. And experience has shown that it is an amazingly effective tool in a wide variety of applications; the Swedish National Academy of Sciences does not hand out the Nobel Prize for nothing. An investigator just has to develop his or her technique . . .

REVERSE TRANSCRIPTION: MAKING DNA FROM RNA

Of course, DNA serves as the primary library of genetic information for any organism, and it resides primarily in the nucleus of a cell (at least with eukaryotes). But RNA serves as the working transcript of genetic information, and it is from RNA templates that proteins are eventually synthesized in the ribosomes. RNA molecules tend to be much smaller than DNA molecules, they move freely about the cell, and they tend to have short lifetimes (RNA is sometimes described as being like a photocopy of an individual page of an organism's book of life.) Cellular RNA is always single stranded, and it is somewhat less stable physically and chemically than double-stranded DNA (except in certain places it forms "hairpin" double strands). Plus it is quickly degraded by ubiquitous enzymes called RNAases; these continuously get rid of used RNA and recycle it in vivo, but they can also quickly chew up an investigator's target DNA, especially after a cell has died and before it is fixed.

In all life forms, whether prokaryotic or eukaryotic, the direction of transcription is almost always DNA to RNA (the permanent record to the working copy), and very rarely does transcription occur in the reverse

direction. However, some microorganisms, namely retroviruses, contain an enzyme called reverse transcriptase that can do just that—make DNA copies from RNA originals. This enzyme has been isolated and cloned, it is available in many different variations, and its activity can be exploited to make cDNA from RNA in vitro. This ability can be put to great advantage with in situ PCR, because it allows an investigator to indirectly amplify RNA within cells so that it can be readily detected even when the target is in low abundance. This is achieved by first converting single-stranded RNA into double-stranded cDNA with the RT enzyme; then PCR is conducted on the cDNA copy to amplify the sought-after signal (PCR works only with DNA, not with RNA). The result is that an investigator can tell whether a specific gene is being actively expressed within a cell by determining whether RNA copies of the gene are present within the cellular structure (there is an especially elegant way to amplify RNA in a single step, which is discussed on pages 47–48).

All reverse transcriptase enzymes exhibit at least three specific enzymatic activities: reverse transcription, a specific DNA polymerase action, and ribonuclease H activity. The reverse transcription maneuver allows the enzyme to use any RNA as a template to be copied, provided that the action is initiated by an annealed primer at the beginning of the target sequence. The copy being made is always DNA. The ribonuclease H activity serves to peel away the RNA template from the newly created RNA–DNA hybrid; then the enzyme chews up the original RNA molecule into tiny bits. The single-stranded cDNA molecule that remains is manipulated by the enzyme once again; this time, RT acts like DNA polymerase and weaves a complementary strand along single-stranded cDNA to make a double-stranded cDNA molecule.

The different versions of RT enzyme each have slightly different characteristics due to the different origins of the enzymes. Each was derived from a specific retrovirus, which has evolved a particular capsid protein to provide a structural milieu within which the enzyme can do its work. However, in the artificial environment of an in vitro experiment, there is no capsid protein available and hence no optimized milieu for the enzyme. Therefore, the investigator must take special care in using RT enzymes, otherwise various untoward biochemical characteristics of the enzyme can be summoned forth.

In particular, RT enzymes in vitro have a tendency to give up on reverse transcription too early and start chopping up the RNA template be-

fore transcription is complete. This destroys the RNA signal before a complete cDNA copy is synthesized. An investigator minimizes this effect by optimizing reagents, particularly manganese ion and a specific detergent that mimics some of the characteristics of the capsid protein. Generally, vendors of RT enzymes supply an optimized buffer solution containing proper concentrations of manganese ion and detergent—be certain to always use the optimized buffer with each RT enzyme.

Last but not least, there are two newer RT enzymes available with special characteristics. Superscript II, available from BRL-Life Sciences (Gaithersburg, Md.) is particularly adept at reverse transcribing longer segments of RNA (up to 3 kilobuses). The second, r*Tth*, is available from Perkin Elmer (Norwalk, Conn.) and can serve not only as the RT enzyme but also as the thermostable DNA polymerase for PCR, allowing RT and PCR reactions to be carried out in the same buffer in one thermal cycling regime.

HOW TO DESIGN PRIMERS

One of the most important keys to performing PCR successfully is designing a proper primer pair, and then optimizing the annealing temperature so that amplification can proceed smoothly. Fortunately, numerous resources and computerized tools are available to assist in these matters, and an investigator is well advised to pay particular attention to these details early in the development of an experimental protocol, for it will save much agony later on.

Primers for DNA Targets

As described in the previous section on PCR, primers are synthetic oligonucleotides, typically between 18 and 22 bases in length. Some might consider an 18-mer primer a little short, that there is too much chance random annealing might occur. Others would argue that a 22-mer primer is extravagantly long, as the price of a synthesized primer often increases significantly around 20 bases. It is our experience that primers in this range work effectively.

Among the four primary nucleotides (ie, G, A, T, and C) in DNA and primers, two (G and C) are "stickier" than the others, as they form six

hydrogen bonds between them, while A and T form only four. This varying character can affect performance of the primer substantially. Therefore, it is desirable to have G's and C's at the 3′ (downstream) end of the primer, because this will facilitate annealing. However, one does not want a triple GGG or CCC at the 3′ end because this combination is too sticky. Nor should you have AAA or TTT. Rather, the ideal sequence is to have two GC nucleotides followed by an AT type at the 3′ end, like GCT or GGA. The overall GC-content of the primer should be between 45 and 55%.

The two primers should be designed so that they have approximately the same annealing temperature. They should also be designed so that they do not form intrastrand or interstrand base pairs. Single strands of DNA can twist and form loops, such that part of the primer can anneal to a target, then another part can anneal to a spot on the target tens or hundreds of bases down. One must be careful to select sequences that have negligible complementary regions, even in short fragments, in the larger segment of DNA intended as the target. Primers that anneal to multiple regions of the target will neither identify the targeted sequence nor extend properly.

Furthermore, primers should never be complementary to one another, particularly around their 3′ ends. If they complement in this region, they often anneal to each other and thus form *primer dimers*. These get extended by the polymerase into double-stranded DNA the length of two primers combined, minus the length of the complementary region. This is always undesirable, because it consumes the primers and greatly lowers the efficiency of amplification.

Fortunately, there are computer programs available that can figure out these factors (as well as many other more subtle ones), and they are well worth the investment. This software is described briefly below and in detail in Appendix 1.

Primers for RNA Targets

If you want to amplify RNA targets, the design of primers becomes somewhat more complex. Four strategies for RNA amplification are possible. The first three techniques begin with destroying all the genomic DNA with an RNAase-free DNAase treatment to eliminate all DNA

copies of a gene that could lead to false-positive results regarding an RNA target. The DNAase is then inactivated, and one can convert all mRNA to cDNA with an RT reaction by using one of two types of primers.

The first type of primer is called an oligo d(T) primer. All mRNA molecules are single stranded, and they have a poly(A) tail, meaning there is a long series of A's (AAAAAAAAA . . .) at the 3′ end of the RNA molecule. An oligo d(T) primer is simply a long series of T's, which will anneal to the poly(A) tail. When a reaction is performed with reverse transcriptase enzyme, the enzyme will extend the primer, making a complementary copy of the mRNA. But the RT enzyme synthesizes DNA from the RNA template, substituting thymine for uracil, and this reverse transcribed DNA is known as cDNA. If the reverse transcription reaction is properly carried out, all the mRNA will be converted to the more stable cDNA, which is then available for amplification through conventional PCR techniques.

The second type of primer is called a random primer. Random primers are sets of short oligos of random sequence, generally hexamers (only 6 base pairs long). They anneal to complementary strands of mRNA, and the RT enzyme extends them in a manner similar to that described for oligo d(T) primers. Since any hexamer represents a common sequence due to its short length, and since so many types of hexamers are included in a random primer set, essentially all of the mRNA gets converted to cDNA by this method (but not necessarily the early parts of every sequence).

Next, you can use a specific primer to reverse transcribe only the gene of interest from the mRNA rather than all mRNA, as with the oligo d(T) and random primers. The cDNA copy can usually be created using the downstream (antisense) primer for the subsequent PCR reaction, which results in unbounded transcription of the downstream target.

For the last type of RT reaction, please bear in mind that cDNA, which represents a copy of mRNA, is fundamentally different from genomic DNA, because it represents only the *expressed* sequences of DNA. Therefore, cDNA will be missing all of the introns and controlling regions of DNA that are found in the genomic copies, which in fact compose more than 90% of most eukaryotic genomes.

An investigator can exploit this fundamental difference to design special, RNA-specific primers that span introns in the genomic DNA, elimi-

nating the need for the oligo d(T) primers and the wholesale conversion of mRNA to cDNA (or the alternative downstream specific primers). Rather, one designs primers that will anneal only to targeted mRNA sequences by designing the primers so they span introns in the genomic DNA. Then the primers will adhere only to the mRNA templates and the cDNA copies of mRNA, not to any genomic DNA copy of the same gene.

If you combine these special primers with a polymerase enzyme that has both reverse transcriptase and DNA polymerase activity (such as the r*Tth* enzyme described earlier), then you can amplify mRNA sequences directly without going through any specific RT step. This simplifies the whole PCR procedure by eliminating the need for a harsh DNAase treatment as well as a buffer change between the RT and polymerase enzyme steps. Better yet, this procedure allows amplification of two types of nucleotide signals simultaneously (both mRNA and a genomic DNA targets) because there is no need to destroy all the endogenous DNA, and primers for each nucleotide type can be included without interfering with the activity of the other. However, one must know a considerable amount about the sequence of the gene in question to design these RNA-specific primers.

Commercially Available Primer Pairs

Numerous PCR primer pairs are available as stock items from commercial biotechnology companies, such as Synthetic Genetics (San Diego, Calif.), BRL-Life Sciences, and Perkin Elmer. In particular, optimized primers are available for many infectious organisms (such as HIV-1, HSV, HBV, HPVs, etc.) as well as many well-documented human or murine genes (such as *P-53, Bcl, Abl,* and numerous other oncogenes). Use of these stock primers can greatly facilitate application of PCR and in situ PCR. However, optimization work should still be done to ascertain the proper annealing temperatures in the thermal cycler to be used as well as with the type of specimen on which the experiments will be conducted. Furthermore, a researcher must select a primer pair to amplify the proper length of DNA for the particular application; and unfortunately, the optimal primer pair is simply not always available, even with commonly studied diseases and genes. Be-

sides, a knowledgable researcher using the proper software can often design *better* primers.

Length of Desired Amplicon

Recent publications have shown that amplification of genes up to 10 kilobases (kb), even 20 k, is possible. However, this long PCR is not frequently used for in situ work, because the primary purpose of amplification in most circumstances is to *detect* specific genes, not clone them. Some workers, including those in our own laboratory, are experimenting with long PCR on freshly fixed tissues to amplify long sequence in situ, and then microdissecting the amplified tissue to recover lesion-associated abnormal sequences. This sort of amplification works better with fresh or freshly fixed tissues, as often the DNA in older fixed tissue has degraded and broken into shorter segments.

But for most in situ PCR work, relatively short amplicons are used. Our laboratory has had great success with amplicons in the 150 to 500 bare pair (bp) range, and that is what we usually target. The amplicons should not be so small as to be prone to diffusion away from the original locus of the target, nor should they be so long as to lower efficiency of amplification in the difficult environment of a cellular matrix. Other workers, notably Haase (University of Minnesota), have previously argued the benefits of longer amplicons and multiple primer pairs; but our experience has shown that longer amplicons lead to a much lower efficiency of amplification, making the amplified signal much more rarified and much harder to detect. Furthermore, Haase has also reported instances of leakage of amplicon with the multiple primary pairs, and it seems this may be due to difficulty in optimizing one annealing temperature for many different sets of primer pairs simultaneously, such that shorter, fragmented amplicons can develop. Instead, our group finds that diffusion and leakage can be better controlled by proper heat fixation and highly optimized proteinase K digestions rather than relying on the sheer size of an amplicon to keep it in place.

For reverse transcriptase reactions, the length of the section for reverse transcription is unimportant. Rather, we typically use the downstream (antisense) primer for the PCR reaction, (described on pages 43 and 47). Alternatively, you can use oligo d(T) primers, which effectively transcribe all of the RNA into cDNA.

Sources for Sequence Data and Computerized Design of Primers

There are several useful sources for sequence data. First is the scientific literature, particularly if your project follows earlier research on a similar matter. But be aware that errors in the transcription of tedious DNA sequences seem to be common in the published literature.

A much more useful and up-to-date source for sequence information is GenBank, an extensive, freely available database (operated out of the Los Alamos National Laboratory in New Mexico) that is accessible on the World Wide Web through the National Library of Medicine. GenBank has sequence data available for a wide variety of genes from various species, though its collection is most extensive with human, primate, and rodent species. GenBank can be contacted through the Web or via modem, and sequences can be downloaded digitally, with little or no transcription error.

The data are most useful if used in combination with a software that is specially designed to process these data and select primer sequences, after various desired characteristics for the primers (or hybridization probes) have been input. A typical program is *RightPrimer* (BioDisk Software, San Francisco, Calif.), which we have found to work quite effectively. Many colleagues sing the praises of *Oligo* (National Biosciences, Inc.; Plymouth, Minn.), though it is perhaps not as economical as the former package. A number of other software companies offer similar programs, including Kodak, DNAStar, Clontech, Hasting, IntelliGenetics, NBI, W. R. Engels, and Genetics Computer Group (see Betsch and Talbot 1995). Freeware software on primer design can even be obtained through the Internet.

To use the software you first establish digital contact with GenBank or another database through *Netscape, Mosaic,* or the software offered by GenBank authorities; and a specific genetic sequence is identified by a number of means. Then you download a fairly large quantity of sequence data from GenBank (directly through the Web or via e-mail) and input the data into the primer design software. You then specify various design criteria, such as the minimum melting temperature, the desired length (300 to 500 bp for in situ PCR), and the GC content. The program then evaluates all theoretically possible primers for a given target sequence that has been input and analyzes the possibilities for self-complementarity, secondary priming sites, restriction sites, propensities for forming

loops, secondary structures, etc. The software then proposes designs with scores to reflect overall quality, and it can also make proposals for hybridization probes to identify the chosen amplicon. The investigator then chooses the best-looking primer pairs and probes and orders them up from a supplier. Suffice it to say, this program takes a lot of the guesswork out of primer and probe design, and it can give the novice the skills of a master.

Details on Primer–Probe Design for Computer-hating Luddites

For DNA amplification, we routinely amplify gene segments of 100 to 500 base pairs, and we find this size works well. Regarding the design of primers, be sure to keep the following points in mind:

- Choose a good target sequence (GenBank is the best source for sequence data); if a lot is known about the gene of interest, try to keep the amplified region within a coding region of the gene, as these regions are the most conserved among a population of genomes.
- The length for both sense and antisense primers should be 18 to 22 bp; for probes, we prefer 40- to 80-bp oligonucleotide probes and 100- to 200-bp PCR-synthesized probes (be certain these probes seek an internal region of the amplicon and do not overlap or complement the primer sequences at all).
- At the 3′ ends, primers and probes should be rich in GC-type base pairs to facilitate complementary strand formation (a GC pair will have three hydrogen bonds, and an AT pair will have two; in other words, primers can grab on and anneal more tenaciously if either G or C nucleotides lead the way). Primers should not start with an A or T, nor should they begin with a triple GC-type bond (ie, not GCG or CCC, but GCT or GGA, etc.).
- The preferred *overall* GC content of the primers and probes is from 45 to 55%.
- Try to design primers and probes so they cannot form intrastrand or interstrand base pairs. Furthermore, the 3′-ends should not be complementary to each other (like one being C-G and the other being G-C), or they will anneal to one another and form primer-dimers.
- Try to design primers that are to be used in reverse transcriptase reactions, so that they will not anneal to multiple areas of the RNA

and form secondary structures, like loops. hairpins, etc. (see also Pallansch and co-workers 1990).

- One must make a preliminary estimate of the annealing temperature to have a starting point for optimization reactions.

There is no easy formula to calculate annealing temperature; however, one generally starts with an estimate of the T_m for the primers and probes (midpoint temperature, sometimes known also as melting temperature). This temperature can be determined analytically by measuring absorbance of a standard solution with a spectrophotometer, but most investigators use the following formula or a variation:

$$T_m = 81.5°C + 16.6 (\log M) + 0.41 (\text{percent GC}) - 500/n$$

where n is the length of the primers (omit the $500/n$ factor entirely for any primer or probe less than 100 bp in length) and M is the molarity of the salt in the buffer, usually 0.047 M for DNA reactions and 0.070 M for RT reactions

A simple formula that is sometimes used for primers of 18 bp or less is the following:

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

If using AMVRT, the T_m value will be lower, and the following formula is often used:

$$T_m = 62.3°C + 0.41 (\text{G + C percent}) - 500/n$$

After calculating the T_m use the rules outlined below to estimate initial annealing temperature.

ANNEALING TEMPERATURES FOR PRIMERS AND PROBES; TOUCHDOWN PROTOCOLS

Whether you are using primer–probe design software or using the formulas given above to calculate T_m, you must make an estimate of what the ideal annealing temperature would be. Usually, we find optimal primer annealing to be at 2° to 4°C below its T_m and the optimal probe

annealing to be 5° to 8°C below its T_m for 40- to 80-bp oligo probes, and 8° to 10°C below its T_m for longer PCR-synthesized probes. However, these estimates provide only an approximate temperature for annealing, since base stacking, near-neighbor effect, and buffering capacity may each play a significant role for any particular primer or probe.

Please bear in mind that the highest fidelity annealing occurs at relatively high temperatures for any primer or probe, and maximum specificity goes hand in hand with high fidelity. If the annealing temperature is too low, spurious priming can occur, where the match between primer and template is not exactly complementary. Nonetheless, annealing occurs anyway, because DNA has an enormous affinity for being double-stranded at lower temperatures, even when there are base pair mismatches. Then these false primes can get extended by polymerase enzyme, and spurious amplification products can result. On the other hand, if the annealing temperature is too high, there is little or no annealing of the primers to the template. Then there is no place for the polymerase enzyme to grab on for extension and little or no amplification of any kind results.

Second, annealing temperatures are important because in situ reactions in general are neither as robust nor as efficient as solution-based ones. We hypothesize this is because large numbers of primers cannot easily reach DNA templates inside cells and tissues, because numerous membranes, folds, the tissue matrix, and other small structures can prevent primers from reaching complementary sites as readily as they do in solution-based reactions. Thus the temperature of annealing should be just right, to make the best of a difficult situation. In other words, the annealing temperature *must* be optimized.

There is an instrument system we have found to be particularly useful in the optimization procedure. It is a thermal cycler called a Robocycler Gradient (Stratagene; LaJolla, Calif.). This instrument is specially designed with a gradient annealing block, which allows a range of annealing temperatures to be screened in a single run. Unfortunately, the machine is not capable of performing in situ reactions on glass slides. However, if you have access to such a machine belonging to a colleague, it is particularly useful for this optimization chore.

One last alternative bears mentioning in this discussion on annealing temperature. Many thermal cyclers are capable of performing something known as a touchdown protocol, where the annealing temperature is initially set rather high, but it ratchets down by about 0.5°C with each sub-

sequent annealing step for the first 10 to 20 cycles. The idea here is to first create a number of high fidelity amplicons, which get geometrically amplified in subsequent cycles. In other words, the signal:noise ratio is increased for better results, even though the final annealing temperature might be substantially below the optimum. This trick can be helpful for those who do not have the time to fully optimize the annealing temperature of their reaction, but it is not a good long-term solution to the annealing optimization problem.

CHAPTER 2

PRELIMINARY SOLUTION-BASED REACTIONS

Before you attempt to conduct in situ PCR reactions on tissues or cells, we strongly recommend that you first optimize the molecular biology reactions in solution to get the primers, the probes, and the incubation temperatures all working right, before throwing the additional complication of cellular matrices into the mix. Investigators universally report that amplification reactions are more problematic in tissues and cells, and virtually no one gets in situ amplification to work right the first time he or she tries (at least if the researcher is working alone). Unfortunately, troubleshooting these early reactions is usually difficult because there are so many variables involved.

Much time, energy, and emotional distress can be saved by getting the molecular reactions optimized *before* moving on to the morphology work. Furthermore, solution-based reactions are relatively easy, and you can have success rather quickly—often on the first try. Then the reactions can be quickly optimized so the results will be even better, and optimized parameters almost always transfer more successfully to in situ protocols. Solution-based reactions can also serve as controls for in situ PCR, so they are a valuable tool to master. Finally, it is often more emotionally satisfying for everyone in the lab to be pursuing a goal they have confidence *can* work, rather than suffering through failed reaction after failed reaction by trying to do too much at one time.

But before DNA or RNA can be amplified in solution, it must be extracted from the cellular matrix in some manner. Several extraction procedures are discussed below.

EXTRACTION OF DNA/RNA USING COMMERCIAL PREPARATIONS

As molecular biology advances, more and more procedures are being simplified by new kits and new reagents that are constantly being introduced and improved. One such product is GeneReleaser (BioVentures, Inc.; Murfreesboro, Tenn.). This reagent, when used in combination with a thermal cycler, is able to extract DNA and RNA from a variety of sources, at least to an adequate degree for use in PCR and RT reactions. The manufacturer offers simple protocols for a wide variety of specimens, including fresh tissue, paraffin-embedded tissue, blood, plant tissue, semen, cell suspensions and bone tissue.

Another set of products specific to RNA extraction are available from Biotecx Laboratories, Inc. (Houston, Tex.; www.biotexc.com). Ultraspec and RNAzol are reagents that can extract total RNA (free of proteins and DNA) from a variety of specimens in as little as 30 min. We have found these products to be useful in our laboratory.

These products, and others like them, save a lot of work in the lab, and we surely recommend their use. However, for those who like to do things the old-fashioned way, the following protocols work perfectly well too.

PROTOCOLS

Extraction of DNA/RNA from Various Cell Suspensions, Including Mononuclear Cells, Other Blood Cells, and Single-cell Suspensions

Peripheral blood monocytes (PBMCs) are first isolated by the density gradient Ficoll–Histopaque method. The layer of PBMCs is drawn off with a sterile transfer pipette and placed in a sterile 1.5-mL loop screw-cap tube. The PBMCs or single-cell suspensions are then washed twice with sterile 1× PBS. About 1 million cells are then aliquoted into each tube. Cells are then pelleted by centrifugation and resuspended in 1×

PBS. About 1 million cells are then aliquoted into each tube. Cells are then pelleted by centrifugation and resuspended in 1× PBS.

DNA is extracted by briefly pelleting the PBMCs or cell suspensions at 1700 *g* for 2 min. The pellets are resuspended in 250 μL 1× TN buffer (from 2× TN stock − 500 μL tris-HCl (pH 8.0), 2 μL 5M NaCl, 47.5 mL dd H_2O) followed by the addition of 250 μL 1× TN buffer + SDS (25 μL 2× TN, 5μL 10% SDS, 20 μL dd H_2O) and 50 μL of proteinase K (1 μg/μL) (Sigma). The tubes are then incubated on a heat block for 15 min at 65°C to digest the proteinaceous material, which then must be removed through solvent extractions. So following incubation, add 500 mL of phenol to the tube (**caution:** phenol can cause burns) and gently agitate for a few minutes at room temperature. Spin at 1700 *g* for 2 min and remove the aqueous layer, being careful not to transfer any sludge from the interface. Put this supernatant into another tube. Add 500 μL of phenol, flicking the tube few times. Spin again, and transfer the aqueous layer to a new tube. Add 500 mL of chloroform to new tube, gently agitate, and spin again. Remove the aqueous layer to a new tube, and add 1 vol (100 μL) of 7.5 M ammonium acetate and 4 vol (400 μL) of chilled (−20°C) 100% ethanol to precipitate the DNA (some people will also add 1 μL of yeast tRNA at 1 μg/μL to enhance DNA precipitation). Gentle agitation should now cause DNA and RNA to precipitate out of solution. Keep the tube chilled for a few minutes, then centrifuge at 1700 *g* at 4°C. Aspirate the supernatant, add 500 μL of 70% ethanol, resuspend the pellet, and spin. Aspirate the supernatant, repeat the 70% ethanol rinse, aspirate the supernatant, and dry by leaving the tube inverted over a Kimwipe for 1 h. The pellets are resuspended in 100 mL of sterile DEPC-treated dd H_2O, and aliquots of this solution can be used for PCR or RT reactions (if this procedure is properly followed, the concentration of the NDA should be in the range of 0.05 μg/μL, but analysis on a spectrophotometer is needed for accuracy better than an order of magnitude).

Extraction of DNA from Paraffin-embedded Tissue

A 10- to 20-μm-thick section of paraffin-embedded tissue (eg, lymph node) is placed on a hard sterile surface, and all excess paraffin surrounding the tissue is manually removed with a sterile razor. The section is then placed in a sterile 1.5-mL tube and incubated at 60°C for 15 min, to

melt the paraffin. The tissue is then washed twice, first with xylene for 5 min and then with 100% ethanol for 5 min.

The deparaffinized tissue prepared in such a manner is cut into the smallest possible pieces with a new sterile razor blade and then placed in a sterile 5.0-mL tube (VWR) containing 2.7 mL of extraction buffer (10 mM tris-HCl (pH 8.0), 100 mM NaCl, 2% SDS, 10 mM EDTA) (1) and 300 μL of proteinase K (1 μg/μL) The tube is incubated in a 42°C water bath for 12 to 18 h. Then 500 μL of fresh proteinase K is added, and the digestion is allowed to proceed for an additional 8 h. The tube is centrifuged at 8000 rpm for 30 min at 4°C. Then the DNA is isolated as described above with a phenol–chloroform extraction and an ethanol precipitation.

Extraction of DNA from Bone Fragments or Forensic Material (Example: Desiccated Tooth)

Intact teeth can preserve DNA for long periods of time after death (centuries or more), even in adverse conditions. Furthermore, the structure of the tooth keeps the endogenous DNA from being contaminated with external DNA, as might otherwise happen in a mass burial site, for example. This DNA can often provide a wealth of information about the individual and the culture in which the individual lived.

A human tooth is first scrubbed and thoroughly rinsed with dd H_2O to remove any dirt and debris. The tooth is split vertically to expose the pulp cavity. The pulp is scraped into a sterile 1.5 mL tube.

The dental pulp is then subjected to treatment with InstaGene DNA Purification Matrix (Bio-Rad Laboratories, Hercules, Calif.) as described by the manufacturer. The DNA thusly obtained is concentrated by the phenol–chloroform extraction and ethanol precipitation procedure as described above. The dried pellet is resuspended in 50 mL of DEPC-treated dd H_2O and aliquoted for further analysis.

SOLUTION-BASED PCR PROCEDURES

The amplification cocktail we are recommending here for solution-based reactions is the same one we will later recommend for in situ reactions, so this protocol is slightly different from standard PCR protocols (eg, it

contains gelatin). We are trying to minimize the variables to simplify later troubleshooting. As each investigator optimizes his or her protocol through these preliminary reactions, the modifications should be brought through as the optimized parameters for the in situ reactions.

Protocol: Solution-based DNA PCR

Create amplification reactions containing the following: 1.25 μM of each primer, 200 μM (each) dNTP, 10 mM tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 0.1 μg DNA template, and 0.1 unit/μL *taq* polymerase. The reaction should be run parallel in four tubes, with two tubes serving as duplicate experiments, another as a positive control with known DNA template, and the last as a negative control with no primers. The following is a convenient recipe for a master mix:

10 mM each dNTP	10.0 μL
1.0 M tris-HCl (pH 8.3)	1.0 μL
1.0 M KCL	5.0 μL
100 mM $MgCl_2$	2.5 μL
0.01% gelatin	10.0 μL
dd H_2O	57.5 μL
Total Volume	*86 μL*

1. Pipette 21.5 μL into each of four identical tubes that will fit the tube-type thermal cycler that is to be used (usually 0.5- or 0.2-mL tubes).
2. Into *each* of the two experimental tubes, pipette in addition:

25 μM forward primer (SK 38 for HIV-1)	1.25 μL
25 μM reverse primer (SK 39 for HIV-1)	1.25 μL
0.05 μg/μL DNA template	0.5 μL
taq polymerase (Ampli-Taq 5 μ/μL)	0.5 μL

3. Into the positive control tube, pipette in addition:

25 μM forward primer (SK 38 for HIV-1)	1.25 μL
25 μM reverse primer (SK 39 for HIV-1)	1.25 μL
0.05 μg/μL known DNA sample (see note)	0.5 μL
taq polymerase (Ampli-Taq 5 Units/μL)	0.5 μL

Note: A known DNA sample would typically be a plasmid copy of the gene of interest.

4. Into the negative control tube, pipette in addition:

25 μM forward primer (SK 38 for HIV-1)	1.25 μL
2.5 μM reverse primer (SK 39 for HIV-1)	1.25 μL
0.05 μg/μL known DNA sample (see note)	0.5 μL
dd H_2O (in lieu of enzyme)	0.5 μL

Note: The same known DNA as used in the positive control.

5. Into each tube, put one drop of heavy mineral oil to serve as a vapor barrier during thermal cycling (this may not be absolutely necessary if you are using a thermal cycler with a heated lid apparatus; however, oil atop the reaction more closely mimics the conditions of in situ PCR).

6. Run 30 cycles of the following amplification protocol:

Denaturation	94°C	30 s
Annealing	45°C	1 min
Extension	72°C	1 min

The annealing temperature of 45°C is optimal for HIV-1 primers SK-38 and SK-39. However, it is rather low for most protocols and may not result in high fidelity annealing or high specificity with other primer pairs. Rather, you should start with a better estimate for annealing temperature for the primers used, which can often be obtained through primer design software, from the group that synthesized the primers, or from a variety of other sources.

7. After amplification, remove the tubes from thermal cycler and remove completely the mineral oil from the top surface. This can be done quite easily by removing as much oil as possible with a micropipette tip, then transferring the remaining contents to a parafilm surface. Push the drop of fluid along the parafilm and the remaining oil will be absorbed. A micropipettor can then recover remaining drops.

8. Electrophorese PCR products on a 1.5% agarose gel at 100 V for 1 to 2 h, along with lambda or some other standard ladder for scale. Stain gel with ethidium bromide and transilluminate. There should be strong

bands in the two experimental and in the positive control lanes, exactly at the proper size for the target DNA fragment. The negative control should have no distinct bands, rather just some fuzziness at the end of the lanes where the unamplified primers end up. Be sure to take a photo of the gel with a photo documentation system to compare with later runs and to help gauge the degree of optimization.

If the gel shows no distinct bands in any lane, then the PCR amplification either failed completely or it was so poorly optimized that not enough DNA was synthesized to register a band in a gel. You should verify the quality of the reagents, the accuracy of the thermal cycler, the validity of the primer sequences, etc. It is also possible that the annealing temperature was too high or the denaturation was improper. If there are multiple weak bands in the experimental lanes and in the positive control, then it is likely that false priming is occurring, and the annealing temperature should be raised. If there is a strong band in the positive control and weak bands or no bands in the experimental lanes, then there may be a problem in the DNA extraction technique, or perhaps there was poor efficiency of amplification. If there are fairly bright, fuzzy bands at about 50 bp, near the end of the experimental and positive control lanes but not the negative control. then primer dimers may be forming, and the primers should be redesigned.

You need to play with the various parameters, particularly the annealing temperature, until strong, distinct, single bands appear in both the positive control and experimental lanes. Then the protocol is optimized.

Adaptations for Solution-based RNA PCR

In later chapters, there are detailed discussions of in situ PCR for RNA targets. Two methods are described, and either can be adapted to solution-based reactions just as the basic DNA reaction was adapted above. It is even more important to optimize with these solution-based reactions for RNA targets, because there are simply more steps to the procedure (and more things to go wrong), and the overall reaction is usually not quite as robust as with DNA targets.

In particular, two methods of PCR for RNA targets are described. The first begins with a DNAase treatment to eliminate all endogenous DNA, followed by a reverse transcriptase reaction with oligo d(T) primers to convert all mRNA to cDNA. The targeted sequence is then amplified

from cDNA. The second involves using a special DNA polymerase enzyme that also has reverse transcriptase properties. Special RNA-specific primers are used that span introns in the genomic DNA, and the RNA targets are reverse transcribed into cDNA and then amplified using the same buffer and the same enzyme. No DNAase treatment is used, so DNA targets are still available for amplification simultaneously.

With either of these methods, optimization in solution-based reactions is helpful. Reverse transcription can be tricky business (as described earlier), and if you are using the more complex intron-spanning RNA-specific primers, then it is necessary to verify that the primers work in solution before attempting to use them in the even more tricky environment of the in situ realm. The matter becomes doubly complex if you are trying to amplify RNA targets and DNA targets simultaneously. Believe us, all these cool things are possible—but practice surely helps.

VERIFYING PROBES AND DETECTION SYSTEMS

The most successful PCR amplifications are worthless if the probing or detection systems fail. Therefore, it is useful to test these components in a simpler context before jumping into hybridizations to nucleic acid targets within cells or tissues. A good testing procedure would be to make Southern blots on the gels above using the appropriate probes and detection systems (see "Southern Blot Alternative," below). However, blotting can be somewhat tedious and time-consuming, and it introduces new variables. Thus we recommend testing probes and detection systems using a simpler dot blot procedure, which also gives the advantage of some quantitative results.

Protocol: Dot Blot Testing of Probes and Detection Systems

1. Run an optimized solution-based PCR reaction in four tubes, as described above (ie, two experimental tubes, one positive control tube, and one negative control tube).

2. Transfer 9 μL aliquots from each reaction result to the new tubes (the remaining cocktail can be run on a gel to verify products, if desired.)

3. To each new tube, add 1 μL of 3 M NaOH to a final concentration of 0.3 to 0.4 M NaOH (this is to denature the DNA). Incubate for 1 h at 60° to 70°C; then cool to room temperature.

4. To each tube, add 10 μL of 2 M ammonium acetate (pH 7.0) to a final concentration of 1 M. Make four serial 10× dilutions of the contents of each tube, for a total of 20 tubes (4 tubes times 5 concentrations).

5. Cut a nitrocellulose–nylon membrane to an appropriate size. Mark the membrane so that a gridwork of 4×5 can later be identified (use a black ball-point pen; Paper Mate's ink will not run in later hybridization solutions). Thoroughly wet the membrane in dd H_2O for at least 5 min. Place the membrane atop a clean filter paper.

6. Place 1 μL dots of fluid onto the membrane from the 20 tubes of the optimized solution-based reaction. Air dry the membrane.

7. Cross-link the DNA samples to the membrane by using a uv light box (follow the manufacturer's instructions; typically a 5-min exposure) or bake the membrane in a vacuum oven at 80°C for 1 h (be sure to use a vacuum oven, as nitrocellulose is highly combustible).

8. Place the membrane into a hybridization bag (the Seal-a-Meal style bags are particularly convenient). Prehybridize the membrane by adapting the hybridization solution listed on page 86. To adapt the solution, increase the volumes specified by 10 or 20 times to accommodate the larger membrane and hybridization bag and leave out the probe. Put the adapted solution into the bag (usually 1 to 2 mL), remove any air bubbles, seal the bag, and incubate at 48°C for 1 to 2 h.

9. Make another adapted hybridization solution in the appropriate volume, this time including the carefully designed and labeled probe. Open the hybridization bag, replace the old solution with the new, remove any air bubbles, and reseal the bag. Incubate 4 h at the appropriate hybridization temperature (usually between 45°C and 55°C), preferably on a rocking platform or in a shaking water bath. Leave out the 95°C heat treatment specified for the in situ reactions; nitrocellulose is not stable at that temperature.

10. After incubation, conduct posthybridization procedures by adapting the appropriate protocols for the in situ reactions, as was done above. Bear in mind that it is possible to multiplex the probes and detection systems, so several different targets of either DNA or RNA (which is now amplified cDNA) can be sought at once. Each different probe will require a different detection system, and this will take some experimentation to perfect. If one is multiplexing in this way, it is often helpful to first test each probe-detection system independently and then to try all the systems together to see if there is any interference.

What you are hoping to see after development of the membranes is an appropriate signal corresponding to the appropriate dilutions and controls. If everything turns out negative, then be sure to run the extra samples from Step 2 on an electrophoretic gel, to ascertain whether you had the proper DNA to begin with. If everything turns out positive, then nonspecific hybridization or detection is occurring, and you must carefully troubleshoot the various parameters. However, it is prudent to ascertain whether any problems exist here in this simplified format before going on to the much more complex environment of the cell and trying to troubleshoot there.

Southern Blot Alternative

For some laboratories, the Southern blot is a standard procedure and the investigators are particularly skilled at interpreting these sorts of results. If this is the case, by all means use Southern blots to optimize the probes and detection systems. After electrophoresis, simply blot the gel onto a nitrocellulose or nitrocellulose–nylon membrane, using capillary or electroblotting techniques, and carry through appropriate hybridization and development procedures adapted from the protocols that are intended for use with the in situ procedures.

The Southern blot can offer several advantages over dot blots. First, the Southern blot is an exact replica of the size-separated DNA pattern that would be found in the electrophoresis gel, for which you would already have an independent photodocumented record of results. Second, the Southern blot is much more sensitive than the ethidium-bromide-staining technique, so we can get results from a weak signal with Southern blots that might be totally missed in examining gel bands. Third, and perhaps most important, the Southern blot can give much more specific information as to problems with nonspecific binding, as it will show which size of DNA fragments are associated with nonspecificity. All of these factors can assist in troubleshooting problematic reactions.

CHAPTER 3

PREPARATIONS OF GLASS SLIDES AND TISSUES

Before you can perform in situ reactions by any of the protocols discussed above you must first obtain the proper sort of glass slide (various sources are described in Chapter 8). Then the glass surface must be treated with the optimal silicon compound. Fortunately, as the in situ PCR market develops, slides specialized to this application are becoming readily available from various manufacturers. However, since proper slides are important to the success of the protocol, we wish to discuss the basics in detail.

GLASS SLIDES AND VARIOUS SEALING TECHNOLOGIES

First, you should always use *glass* slides. Not only does the glass withstand well the stress of repeated heat denaturation but it also presents the right chemical surface (silicon oxide) that is needed for proper silanation. Glass also has optical qualities that are without compare.

There are several different ways to seal the slide from evaporation during the thermal cycling regime required for PCR. Several of these techniques are proprietary and some involve specialized equipment; the various options are described in detail on pages 54–56. However, the tried-and-true method that everyone can use involves sealing the slides

with nail polish or varnish, and this is the standard technique that we are using as a common denominator throughout this manual.

If you choose to use the nail polish technique, slides with special printing or Teflon coatings that form individual wells are especially useful. This is because vapor-tight reaction chambers can be formed on the surface of the slides after the cover slips are adhered with coatings of nail polish around the periphery. These reaction chambers are necessary because within them, proper tonicity and ion concentrations can be maintained in aqueous solutions during thermal cycling—conditions that are vital for proper DNA amplification. The Teflon coating serves a multiple purpose in this regard. First, the Teflon helps keep the two glass surfaces slightly separated, allowing for reaction chambers about 20 μm in height to form between. Second, the hydrophobicity of the Teflon combined with the pressure applied by a cover slip helps spread small volumes of reaction cocktail over the entire sample region, without forcing much fluid out of the periphery or allowing air bubbles to develop. Third, the Teflon border helps keep the nail polish from entering the reaction chambers when the polish is being applied; this is important, for any leakage of nail polish into a reaction chamber can compromise the results in that chamber. Last, the Teflon border allows several chambers on one slide (typically three) so you can run two controls on a slide simultaneously with an experimental reaction.

AES SILANATION: PUTTING ON THE POSITIVE CHARGE

Normally, when you silanate glass slides, it is for the purpose of making the slide surface hydrophobic. However, the AES silane that is specified in this protocol has quite a different surface effect. This silicon compound imparts a strong, positive, and persistent electrical charge, to the slide by forming aminopropyl derivative of glass at the surface of the slide. The resulting positive charge, and electrostatic attraction cause the cells or tissues to adhere with great tenacity throughout the PCR-hybridization procedure. Experiments have been conducted with slides coated with alternative adhesives, including white glue, albumen, chrome gelatin, and poly-L-lysine. However, we have found that slides treated with AES silane have superior tissue adhesion and lower background

with all tissue types tested, including mixed-cell suspensions, paraffin sections, and frozen sections.

It must be noted that many slide vendors offer special coatings that seem to be just as effective as AES silane. In fact, we suspect that many of these special coatings are AES silane, but the manufacturers just will not tell us.

To prepare glass slides properly, follow this procedure:

1. Prepare the following 2% AES solution just before use:

3-aminopropyltriethoxysilane (AES: Sigma A-3648)	5 mL
Acetone	250 mL

2. Put the solution into a Coplin jar or glass staining dish, and dip the glass slides in 2% AES for 60 s. Set aside for 1 to 2 h (see Chapter 8 for sources of both Coplin jars and the proper *unsilanated* glass slides).
3. Dip the slides five times into a different vessel filled with 1000 mL of distilled water.
4. Repeat Step 3 three times, changing the water each time.
5. Air dry in a laminar-flow hood from a few hours to overnight; then store the slides in a sealed container at room temperature. Try to use the slides within 60 days of silanation; 250 mL of AES solution is sufficient to treat 200 glass slides.

PREPARATION OF TISSUE

Cell Suspensions

To use peripheral blood leukocytes, first isolate cells on a Ficoll–Hypaque density gradient. Tissue culture cells or other single-cell suspensions can also be used. Prepare all cell suspensions with the following procedure.

1. Wash the cells with 1× PBS twice.
2. Resuspend the cells in PBS at 2×10^6 cells/mL.
3. Add 10 μL of cell suspension to each well of a silanated slide using a P20 micropipette, and spread it across the well surface.
4. Air dry the slides in a laminar-flow hood.

Cells Cultured on Slide

We have successfully performed the in situ amplification procedure on cells cultured on two different designs of slide. The first is a 4- or 8-well tissue culture slide from Nunc, (Naperville, IL) and the second is a similar Teflon-printed slide from Erie Scientific (Portsmouth, NJ). If you are using the Nunc slides, make certain they have a glass base, as the plastic slides will melt during the in situ amplification procedure.

Sealing of the Nunc slides for thermal cycling can be a little tricky. These slides have a rubber gasket, which should be left on, but protruding parts of the gasket must be shaved off to fit the cover slip. We also reinforce the gasket by applying a layer of nail polish at the junction of the gasket and the glass slides. Unfortunately, occasional leakage still occurs.

The alternative is to use the Teflon-printed slides, as with the other procedures described in this book. However, the slides must be sterilized after silanation and before inoculation with the culture. This is achieved by soaking the slides in absolute ethanol for 30 min and air drying. Then the cells are placed on the slides and cultured overnight in a clean tissue culture incubator (typically humidified, 5% CO_2, 37°C) or in a sterile humidified culture box.

Paraffin-fixed Tissue

Routinely fixed paraffin tissue sections can be amplified quite successfully—even archival tissues. Analysis of sections permits the evaluation of individual cells in the tissue for the presence of a specific RNA or DNA sequence, even if there is as little as one copy per cell (if you have good technique).

For this purpose, tissue sections are placed on a properly prepared slide, as described above. If you are using Teflon-coated slides with nail polish sealing, it is often helpful to use slides that have large single wells (see Chapter 8 for more information), and you use two slides with two Teflon-coated sides facing one another rather than a slide and a cover slip. This allows for a double-thick reaction chamber, which can hold a bit more amplification cocktail. If you are using any of the newer slide systems, follow the manufacturer's instructions.

In our laboratory, we routinely amplify placental tissues, CNS tissues, cardiac tissues, etc., which are sliced to a 3 to 5-μm thickness. Other laboratories prefer to use sections up to 10 μm in thickness; but in our expe-

rience, amplification is often less successful with the thicker sections, and multiple cell layers can often lead to difficulty in interpretation due to superposition of cells. However, if you are using tissues that contain particularly large cells—such as ovarian follicles, plant protoplasts, and large neurons—then thicker sections are appropriate.

1. Place the tissue section on the glass surface of the slide.
2. Incubate the slides in an oven at 60°C for 1 h to melt the paraffin.
3. Dip the slides in a xylene solution (EM grade, benzene-free) for 5 min, and then in 100% ethanol (EM grade) for 5 min. Repeat these washes two or three times to rid the tissue of paraffin completely. This step should be performed in a fume hood.
4. Dry the slides in an oven at 80°C for 1 h.

Note: Some laboratories prefer to deparaffinize in three successive baths of xylene, followed by rehydration in graded ethanols (100%, 95%, 70%, 50%). Do not use denatured alcohol (it contains benzene) or reagent alcohol (it contains isopropanol); EM-grade pathology ethanol, diluted to the proper strength, works best.

Plastic Sections

In situ amplification can be performed successfully on plastic sections; however, the sections must not have been cut so thin that all the cells have been cut in half (less than 2 to 3 μm), as this will lead to excessive leakage of signal. First the plastic sectioned tissue must be deplasticized of methyl methacrylate (MMA) by incubating it in four successive baths of fresh methyl celluloacetate ether (MCA) for 15 min each time; then incubate it in three successive baths in fresh acetone for 10 min each time; and then place it in multiple xylene soaks up to 4 h each time. Then the in situ procedure is the same as with other tissues.

Frozen Sections

It is possible to use frozen sections for in situ amplification; however, the morphology of the tissue following the amplification process is generally not as good as with paraffin sections. The cryogenic freezing of the tissue, combined with the lack of paraffin substrate during slicing, simply com-

promises the morphology of the tissue. Usually, thicker slices must be made, and the tissue can "chatter" in the microtome. As any clinical pathologist will relate, definitive diagnoses are made from *paraffin* sections, and this rule of thumb seems to extend to the amplification procedure as well.

The exceptions to the rule are when you wish to use immunohistochemical techniques to detect antigens that are denatured due to formalin fixation or you are dealing with tissues that must be fresh-frozen to preserve fragile, low-abundance RNA messages. In these sorts of circumstance, use of frozen sections is appropriate.

However, for immunohistochemistry, there are new fixatives, such as Permiofix (Ortho Diagnostics), that preserve cell surface antigens in fixed tissue, which allows the use of paraffin sections. These matters are explained further in Chapter 5.

Protocols

Freezing the Tissue When using frozen sections, it is most important to freeze the tissues properly. Of course, every pathologist has his or her own best method; but here are three methods that we have found to be quite successful.

1. Our preferred procedure is to first cut a 1 × 1-cm piece of polystyrene from a sheet about 3 mm thick (or use a disposable polystyrene coffee cup if a sheet is not available). Then cut a slice of tissue about the same size as the polystyrene square but somewhat thicker (up to 2 cm thick). Pour about 2 mL of Tissue-Tek OCT, an embedding media for frozen tissue specimens (Miles Laboratory, Elkhart, Ind.), onto the polystyrene substrate. Lay down the tissue in the correct orientation, and pour another 2 mL of OCT on top, so that the tissue is covered with OCT. Fashion an immersion tool from wire coat hanger so that the polystyrene–tissue sample can rest on a loop of wire; use the wire handle to immerse the tissue. Slowly lower the apparatus and sample into the liquid nitrogen, and the tissue should freeze in less than 30 s. Then the tissue can be loaded into a cryocassette and cut or put into a −70°C or −140°C freezer for storage.

We have found this method to be best if the tissue does not have to be stored for long periods of time. The polystyrene substrate keeps the tissue from bending during freezing, the OCT solution facilitates cutting and helps preserve morphology, and the rapid freezing prevents the formation of ice crystals.

2. Another method that is almost as good as the first one is to place the tissue into a small zipper-locking bag designed for immersion into liquid nitrogen; these bags are common items in pathology laboratories. The tissue is simply immersed in the liquid nitrogen, and then removed and either loaded into a cryocassette for cutting or put into a −70°C freezer for storage. This method results in good morphology; however, the tissue usually warps during freezing, so smaller sections must often be cut. This is the better method than the method discussed above if there is a need for long-term storage, as the tissue will remain well preserved for years in a −70°C or lower temperature freezer without forming ice crystals or suffering chemical deterioration due to embedding solutions. Special ink pens are available to label these bags so your progeny can figure out what the samples are decades from now.

3. If liquid nitrogen is not available, tissue prepared with polystyrene and OCT (as in method 1) can be wrapped in aluminum foil and placed on dry ice for 10 to 15 min before storing in the deep freezer. However, some ice crystallization in the tissue may occur with this method.

Under no circumstance attempt to freeze the tissue by merely placing it into a −70°C freezer. This will result in an abundance of ice crystals, and the sections will not be suitable for in situ procedures. For example, brain tissue preserved in this manner will show numerous gaps within the tissue section, making analysis difficult at best.

Sectioning the Tissue To conduct in situ amplification, it is necessary to use as thin a section as possible without cutting the cells into halves; for most tissues it is usually down to 5 to 6 μL in thickness. Difficulties will often be experienced in slicing frozen sections thinly, because the tissue is either too cold or it is insufficiently frozen. Usually, it is the latter problem, and this is manifested by a "gooey" area in the middle of the specimen, which will not slice properly. This is remedied by use of pathologist's freezing spray: merely blast the central area with a few quick bursts of spray, wait a few moments, and proceed.

If instead the tissue will not slice at all, it could be that the tissue is too solidly frozen. This is a common difficulty with tissue that has been removed from long-term storage in a −135°C freezer or in liquid nitrogen. To remedy this problem, allow the tissue to equilibrate overnight at −28°C in the cryostat.

Once the tissue is properly sliced, apply it to a slide, dehydrate it for 10 min in 100% methanol, and let it air dry in a laminar flow hood. Then proceed to the heat treatment step described in the basic in situ protocol in Chapter 4.

Archival Tissue

In the Laboratory of Central Nervous Systems Studies at the National Institute of Neurological Disorders and Stroke, Isaacson has successfully amplified RNA and DNA signals in paraffin-embedded brain tissue up to 25 years old. The original fixation methods are generally not known, but Isaacson suspects that formalin was the most common fixative for tissues of this age (for further discussion of fixatives, ancient tissues, and amplification in solution, see Shibatta, 1994 and Höss, Handt, and Pääbo, 1994.

Isaacson uses tissues sliced to a thickness of 5 μm, with a total area of 3 cm^2, which he puts on conventional, silanated slides so that the tissue will fit under a 20 × 30 mm cover slip. Isaacson's amplification protocol is similar to the one described here.

In our own laboratory, we routinely amplify archival sections of somewhat more recent vintage—even material that has been already mounted on slides for some time. To do this, you remove the cover slip and mounting by soaking them in xylene, then deparaffinize as described on page 33. Since the tissue is already on a slide, you do not have a choice about the area of the slide covered by the section or what type of surface preparation to use. Rather, just take a silanated slide, either a single-well or three-well version, and use that as the cover slip. Load the reagents into the wells of the coated slide, place an inverted archival slide on top, and seal around the periphery with nail polish as described later. Or perhaps you could use the new Self-Seal reagent [MJ Research, Inc., Watertown, Mass. **(www.mjr.com)**] to seal the slides. We use either method; perhaps not all of the archival tissue will be subject to the PCR-hybridization procedure, but usually there is a sufficient area to get adequate results.

CHAPTER 4

IN SITU PCR: DNA AND RNA TARGETS

BASIC PREPARATION, ALL PROTOCOLS

For all sample types and all target sequences, the following steps comprise the basic preparatory work that must be done before any PCR-hybridization procedure.

Protocols

Heat-stabilization Treatment for DNA and RNA Messages

1. Place the slides with adhered tissue on a heat block at 105°C for 5 to 120 s to stabilize the cells or tissues.

To achieve maximum specificity and sensitivity, we find this step to be important. We have experimented often with parallel reactions in which the heat treatment has been omitted; in virtually all cases, the strength of the contained signal was decreased and the background was increased. We hypothesize that this sudden flash of heat serves both to immobilize the tissue on the slide and to alter various structures within the cells. Probably a fair amount of cross-linking is going on, and subsequently, the various proteins and membranes are better able to hold the soon-

to-be-synthesized amplicons at the exact loci within the cells where the messages originated. Furthermore, leakage of signal out of message-positive cells is greatly reduced, apparently due to changes in membrane porosity.

Some investigators argue that RNA messages may not be sufficiently stable at these high temperatures to maintain their integrity through a 105°C heat treatment. We wholeheartedly disagree. In fact, our experience is almost the opposite: this heat treatment actually *improves* the stability of RNA messages. The RNA molecule itself is not inherently unstable (at least not in most of its manifestations). Rather, there is an abundance of RNAases about, particularly in the cellular milieu, that can chew up diffuse RNA signals quickly. By flashing the tissue with high heat, these RNAases are largely inactivated or at least compromised, which allows a weak RNA signal to remain intact long enough for reverse transcriptase to make a stable cDNA copy.

However, the various organelles and compartments within which mRNA molecules reside seem to be more fragile than the nuclei where most DNA molecules hang out. We hypothesize that too long a heat treatment tends to encapsulate or cross-link RNA molecules with the proteins with whom they are so intimately involved inside these organelles. Precisely optimized proteinase K digestions are then required to free the RNA molecules from the grip of these cross-linked proteins to make the RNA message available for reverse transcription. To avoid excessively exacting digestions, we simply use shorter heat treatment times (5 to 30 s) with RNA messages, to help prevent RNA–protein cross-linking or trapping of RNA inside highly convoluted splicosomes or other denatured organelles and membranes. We use longer incubation times (90 to 120 s) with DNA messages, with which cross-linking (among proteins at least) is more desirable.

In most circumstances, this heat stabilization step can benefit from optimization of its period. However, we find this step to be absolutely critical for maximum sensitivity and containment of signal, so do not skip the heat treatment.

Fixation and Washes

1. Place the slides in a solution of 2% paraformaldehyde in PBS (pH 7.4, prepared less than 2 weeks previously) overnight at room temperature. Use of Coplin jars or glass staining dishes facilitates these steps.

2. Wash the slides once with 3× PBS for 10 min, agitating periodically with an up-and-down motion.

3. Wash the slides with 1× PBS for 10 min, agitating periodically with an up-and-down motion. Repeat twice with fresh 1× PBS.

4. At this point, slides with adhered tissue can be stored at −20° to −70°C until use. Before storage, dehydrate with 100% ethanol. **Note:** Some investigators prefer a graded series of ethanol before cold storage (ie, 50%, 70%, 95%, 100%), but we always make a beeline straight to full-strength alcohol. However, you may wish to use the reverse series of ethanol to rehydrate the tissues before proceeding to the proteinase K digestion.

If biotinylated probes or peroxidase-based color development are to be used (as with streptavidin or digoxigenin) the samples should further be treated with 0.3% hydrogen peroxide in PBS to inactivate any endogenous peroxidase activity. Once again, incubate the slides overnight, either at 37°C or at room temperature. Then wash the slides once with PBS. If one is in a big hurry, incubate slides 1 h with 1% hydrogen peroxide in PBS, followed by multiple washes in PBS.

If other detection systems are to be used, proceed directly to the proteinase K digestion

Proteinase K Treatment Proper attention to the proteinase K digestion cannot be over emphasized: *this is probably the most critical step* in the whole in situ PCR procedure.

1. Treat samples with 6μg/mL proteinase K in PBS for 5 to 60 min at room temperature or at 55°C (no doubt this represents quite a range, see discussion). To make a proper solution, dilute 1.0 mL of proteinase K (1 mg/mL) in 150 mL of 1× PBS.

2. After 5 min, look at the cells under the microscope at 400×. If the majority of the cells of interest exhibit uniform small, round "bubbles" or "peppery dots" or "salt and pepper dots" on the cytoplasmic membrane (usually about 10 to 20 dots per cell), then stop the treatment immediately with Step 3. Otherwise, continue treatment for another 5 min and reexamine. See Figure 4.1 to see typical cytoplasmic membranes before proteinase K treatment, and Figure 4.2 to see membranes exhibiting peppery dots after treatment.

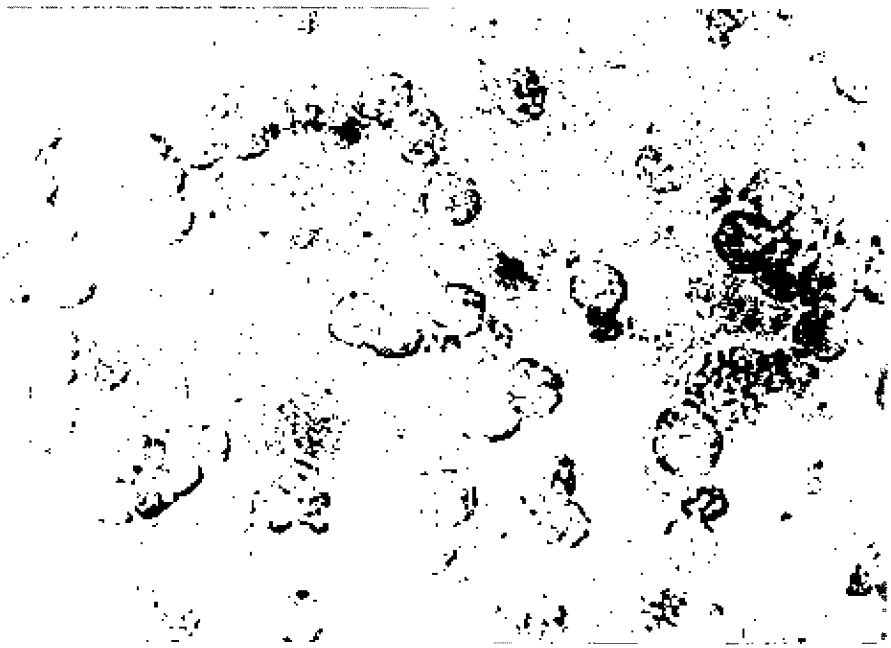

Figure 4.1. Lymphocytes after heat treatment and fixation steps, but before treatment with Proteinase K. Note the smooth cytoplasmic membranes without any "peppery dots" on the cell surface.

3. After proper digestion, heat the slides on a block at 95°C for 2 min to inactivate the proteinase K.
4. Rinse the slides in 1× PBS for 10 s.
5. Rinse the slides in distilled water for 10 s.
6. Air dry.

Alternative Method for Proteinase K Treatment The alternative technique is not generally employed by our laboratory. However, other investigators have found it to be useful, particularly those groups without the services of a pathologist accustomed to quickly analyzing the somewhat subtle morphological changes that are associated with the dots described above.

1. Prepare 6 to 10 extra slides of the specific tissue in question. It is especially helpful if the slides are successive sections or similarly prepared, for the morphology of the various slides must be closely compared later.

Figure 4.2. Lymphocytes after appropriate treatment with Proteinase K. Notice minute cell surface dots. These dots are presumably the result of partial digestion of transmembrane proteins. The dots should be circular, uniform size, and fairly evenly distributed.

2. Prepare an equal number of serial dilutions of proteinase K solution, each stronger than the last. For example, in a first round try 1 μg/mL, 2 μg/mL, 4 μg/mL, and 8 μg/mL. In a second round, home in closer on the range of interest.

3. Decide on a standard time and temperature for digestion, for example, 15 min at 37°C. Treat the slides in the serial solutions for this standard period.

4. Stop the digestion by heating the slides on a block for 2 min at 95°C.

5. Counterstain the slides with hematoxylin or another appropriate stain.

6. Observe the slides under a high power light microscope, looking for any morphological change in the cells. Find the concentrations just above and below where there is observable change to the tissues. Repeat Steps 2 to 5 with a narrow range of concentrations. Use the highest

concentration of proteinase K that did not result in significant change as the optimized concentration.

If all the slides showed change in the first round, repeat the process with lower concentrations of proteinase K or shorter incubation periods; if none of the slides showed change, increase the concentrations or incubation times and repeat the procedure until the proper conditions are found.

7. Once the optimized digestion is determined, use these conditions to process all slides of that particular tissue, fixation method, and thickness of section. Any change in these parameters necessitates reoptimization of the digestion procedure.

Discussion of Optimizing Digestion

The time and temperature of incubation should be optimized carefully for each cell line or tissue section type. With too little digestion, the cytoplasmic and nuclear membranes will not be sufficiently permeable to primers and enzyme, and amplification will be inconsistent (or nonexistent). With too much digestion, the membranes and protein structures will lose integrity. Then the amplicons will spread around the cell unnecessarily, and worse still, the amplicons may leak out of the cells and make surrounding cells falsely positive. Other adverse consequences can include high background and poor morphology. Often with excessive digestion, many cells will show pericytoplasmic staining (ie, staining only around the periphery of the cell), which represents leaked signal that has contaminated cells where no positive signal actually exists. *Attention to detail with the proteinase K digestion can often mean the difference between success and failure in an experiment,* and this digestion should be practiced on extra sections by anyone attempting to conduct this protocol for the first time.

In our laboratory, proper digestion parameters vary considerably with tissue type. Typically, lymphocytes require 5 to 10 min at 25°C or room temperature, CNS tissue requires 12 to 18 min at room temperature, and paraffin-fixed tissue requires between 15 and 30 min at room temperature (these times can be accelerated by using higher temperatures of incubation, up to 55°C). However, the periods can vary widely, and the appearance of the dots is the important factor; unfortunately, the appearance of the dots is less prominent in paraffin sections and in neurologic tissues. Nevertheless, the critical importance of the dots should not be underesti-

Figure 4.3. Representative examples of "dots" on two primary cell lines from a fetal brain. Note tiny dots on the cell surfaces.

mated, since an extra 2 to 3 min of treatment will often result in excessive leakage of signal and poor morphology. See Figures 4.3 and 4.4 for representative examples of dots in two primary cell lines from brain tissue.

Regarding the enzyme used for digestion, we have also experimented with other proteinases (such as amylase, trypsin, pronase, and pepsin) instead of proteinase K. These alternatives have proven successful in many circumstances. However, we have found that almost always proteinase K gives better results, no matter the type of tissue or the method of fixation.

Discussion of DNAase Treatment for RNA Targets Exclusively

As described in Chapter 1, there are two methods of detecting an RNA signal. The first and more elegant method is to simply use primer pairs that anneal at the junctions of spliced sequences of mRNA, such that

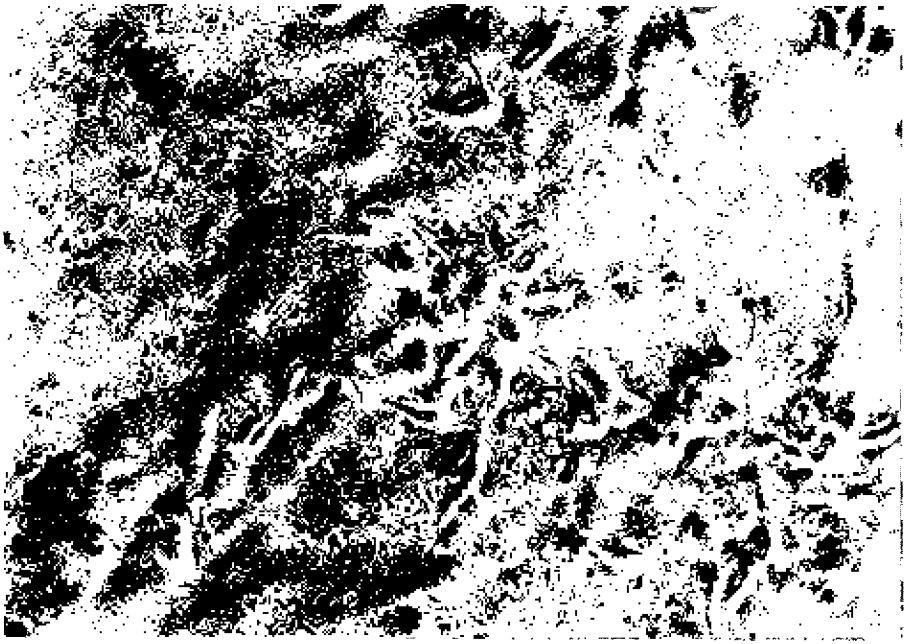

Figure 4.4. Most eukaryotic genes are split into segments, as there are numerous introns in the DNA that are excised during the synthesis of mRNA in the nucleus. This characteristic can be exploited in the design of primers to amplify mRNA signals without interference from the DNA. One can simply design primers so that their sequences flank spliced regions where two exons are fused, such that the homologous annealing sites exist *only* in mRNA and not in DNA. This allows the elimination of a DNAase treatment during slide preparation as well as the simultaneous amplification of both RNA and DNA signals.

the primers would have to span introns within the DNA copies of the gene in question to anneal (which they cannot do). Thus these primer sequences will anneal *only* to messages found in mRNA. By using these RNA-specific primers, one can skip the following DNAase treatment and later amplify both RNA and DNA targets in same specimen. So if this is the strategy being pursued, proceed directly to reverse transcription (or amplification if one is using the r*Tth* DNA polymerase enzyme).

However, often one does not know enough about a gene to make an RNA-specific primer. In that case, the second approach to RT in situ PCR

is to treat the cells or tissue with a DNAase solution subsequent to the proteinase K digestion. This step destroys all of the endogenous DNA in the cells so that only RNA copies, transcribed to cDNA, will survive to provide signals for amplification.

Note: All reagents for use in RNA reactions should be prepared with RNAase-free water (ie, DEPC-treated water). In addition, the silanated glass slides and all glassware should be RNAase-free, which we ensure by baking the glassware overnight in an oven at 250° to 300°C before use in the DNAase or reverse transcription procedures.

Protocols

Prepare a RNAase-free, DNAase Solution

40 mM tris-HCl, pH 7.4
6 mM $MgCl_2$
2 mM $CaCl_2$
1 U/μL final volume of DNAase (use RNAase-free DNAase such as 10 U/μL RQ1 DNAase; catalog no. 776785 from Boehringer)

1. Add 10 μL of solution to each well.
2. Incubate the slides overnight at 37°C in a humidified chamber. When using liver tissue, this incubation should be extended an additional 18 to 24 h.
3. After incubation, rinse the slides with a similar buffer solution that was prepared *without* the DNAase enzyme.
4. Wash the slides twice with DEPC-treated water.

Note: Some cells are particularly rich in ribonuclease, in this circumstance, add the following ribonuclease inhibitor to the DNAase solution: 1000 U/mL placental ribonuclease inhibitor (eg, RNAsin) plus 1 mM DTT. If you are in a rush with any type of tissue, use a higher concentration of DNAase (100 U/μL) and incubate 1 to 2 h. This shorter incubation has the advantages of minimizing any hazards from a slight RNAase contamination of the DNAase reagent.

Reverse Transcriptase Reaction Next, make DNA copies of the targeted RNA sequence so that the signal can be amplified. The following are typical cocktails for the reverse transcriptase reaction.

If using AMVRT or MMLVRT enzyme (for mRNA transcripts less than 1 kb):

10× reaction buffer (see note)	2.0 μL
10 mM dATP	2.0 μL
10 mM dCTP	2.0 μL
10 mM dGTP	2.0 μL
10 mM dTTP	2.0 μL
RNAasin at 40 u/μL (Promega; see note)	0.5 μL
20 μM downstream primer	1.0 μL
AMVRT 20 U/μL	0.5 μL
DEPC treated water	8.0 μL
Total volume	*20.0 μL*

Note: 10× reaction buffer: 100 mM tris pH 8.3, 500 mM KCl, 15 mM $MgCl_2$

Note: RNAsin inhibits ribosomal RNAases; use for optimal yields

If using Superscript II enzyme from BRL (for mRNA transcripts greater than 1 kb):

5× reaction buffer (as supplied with enzyme)	4.0 μL
10 mM dATP	2.0 μL
10 mM dCTP	2.0 μL
10 mM dGTP	2.0 μL
10 mM dTTP	2.0 μL
RNAasin at 40 u/μL (Promega; see note)	0.5 μL
20 μM downstream primer	1.0 μL
Superscript II, 200 U/μL	0.5 μL
0.1 M dTT	1.2 μL
DEPC treated water	4.8 μL
Total volume	*20.0 μL*

Note: RNAsin inhibits ribosomal RNAases; use for optimal yields

1. Add 10 μL of either cocktail to each well. Carefully place the coverslip on top of the slide.
2. Incubate at 42° or 37°C for 1 h in a humidified incubator.

3. Incubate the slides at 92°C for 2 min.
4. Remove the coverslip and wash twice with distilled water.
5. Incubate the slides and 92°C for 1 min, to ensure complete deactivation of DNAase and RT enzymes.

Proceed with the chosen amplification procedure.

Discussion of RT Enzymes

Avian myeloblastosis virus reverse transcriptase (AMVRT) and Moloney murine leukemia virus reverse transcriptase (MMLVRT) give comparable results in our laboratory. Other RT enzymes will probably also work. However, it is important to read the manufacturer's descriptions of the RT enzyme and to make certain that the proper buffer solution is used (which may be somewhat different from that recommended here).

An alternative RT enzyme is available that has low RNAase activity. Called Superscript II, it is available from BRL Life Sciences, and it is suitable for reverse transcription of long mRNAs. It is also suitable for routine RT amplification, and in our laboratory it has proven to be more efficient than the two enzymes described above.

Discussion of Primers for RT Reactions

In our laboratory, we simply use antisense downstream primers for our gene of interest. Other laboratories use both upstream (sense) and downstream (antisense) primers for reverse transcription (ie, same primers used for PCR). However, one can alternatively use oligo d(T) primers to first convert all mRNA populations into cDNA, and then perform the in situ amplification for a specific cDNA. This technique may be useful when one is performing amplification of several different gene transcripts at the same time in a single cell. For example, if you are attempting to detect various cytokine expressions, you can use an oligo d(T) primer to reverse transcribe all of the mRNA copies in a cell or tissue section. Then you can amplify more than one type of cytokine and detect the various types with different probes that develop into different colors (see "Multiple Signals, Multiple Labels in Individual Cells," page 64).

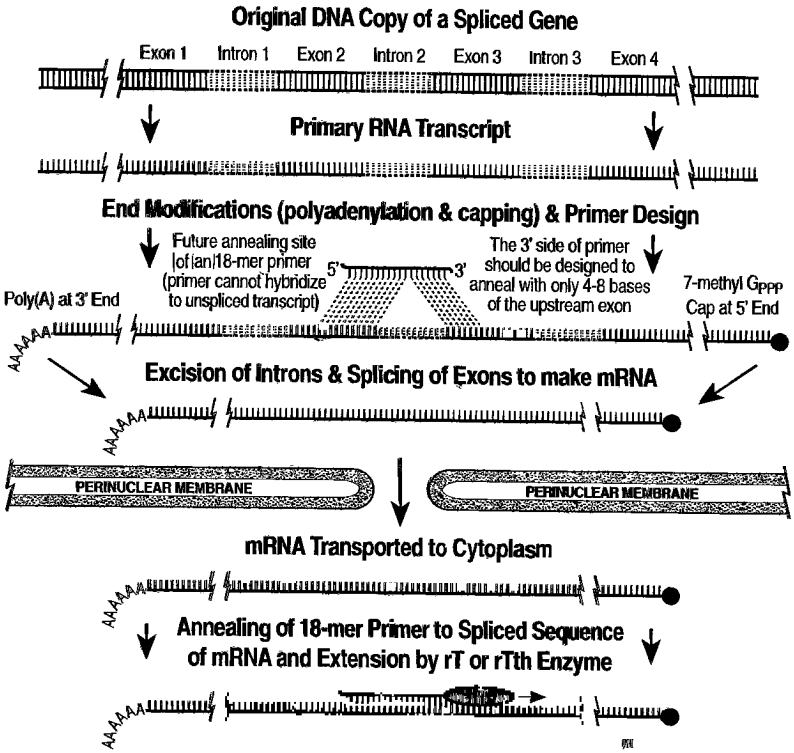

Figure 4.5. Most eukaryotic genes are split into segments, as there are numerous introns in the DNA that are excised during the synthesis of mRNA in the nucleus. This characteristic can be exploited in the design of primers to amplify mRNA signals without interference from the DNA. One can simply design primers so that their sequences flank spliced regions where two exons are fused, such that the homologous annealing sites exist *only* in mRNA and not in DNA. This allows the elimination of a DNAase treatment during slide preparation as well as the simultaneous amplification of both RNA and DNA signals.

In all RT reactions, it is desirable to reverse transcribe only relatively small fragments of mRNA (<1500 bp). Larger fragments may not completely reverse transcribe due to the development of secondary structures or simply because of the peculiar characteristics of RT enzymes (as described on pages 7–8). Many RT enzymes (AMVRT and MMLVRT, at least) are simply not efficient in transcribing large mRNA fragments.

PCR AMPLIFICATION PROCEDURES

Protocols

In Situ PCR with Conventional Sealing Technologies This protocol can be used for all reactions on DNA targets, including procedures following reverse transcriptase reactions where cDNA copies have already been made. However, do not use this protocol for reactions with intron-spanning, RNA-specific primers or with r*Tth* polymerase enzyme, or with reactions where one intends to use Self-Seal reagent (MJ Research) to seal the slides. Optimized protocols for those two variations of in situ PCR are discussed later.

Prepare an amplification cocktail containing the following: 1.25 μM of each primer, 200 μM (each) dNTP, 10 mM tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, and 0.1 U/μL *taq* polymerase. The following is a convenient recipe that we use in our laboratory:

25 μM forward primer (SK 38 for HIV-1)	5.0 μL
25 μM reverse primer (SK 39 for HIV-1)	5.0 μL
10 mM each, 4dNTP	2.5 μL
1.0 M tris-HCl pH 8.3	1.0 μL
1.0 M KCL	5.0 μL
100 mM $MgCl_2$	2.5 μL
0.01% gelatin	10.0 μL
taq polymerase (AmpliTaq 5 U/μL; see note)	2.0 μL
dd H_2O	67.0 μL
Total volume	*100 μL*

Note: Other thermostable polymerase enzymes have also been used quite successfully. However, one should always use the appropriate buffer for the specific polymerase, paying particularly close attention to the optimal $MgCl_2$ concentration.

1. Layer 8 μL of amplification solution onto each well with a P20 micropipette so that the whole surface of the well is covered with the solution. If using a single-well slide for a tissue section, add 12 to 20 μL of the solution to the well. If using gene cones or gasketed slides, follow the manufacturer's instructions. In all cases, be careful to not touch the surface of the slide with the tip of the pipette.

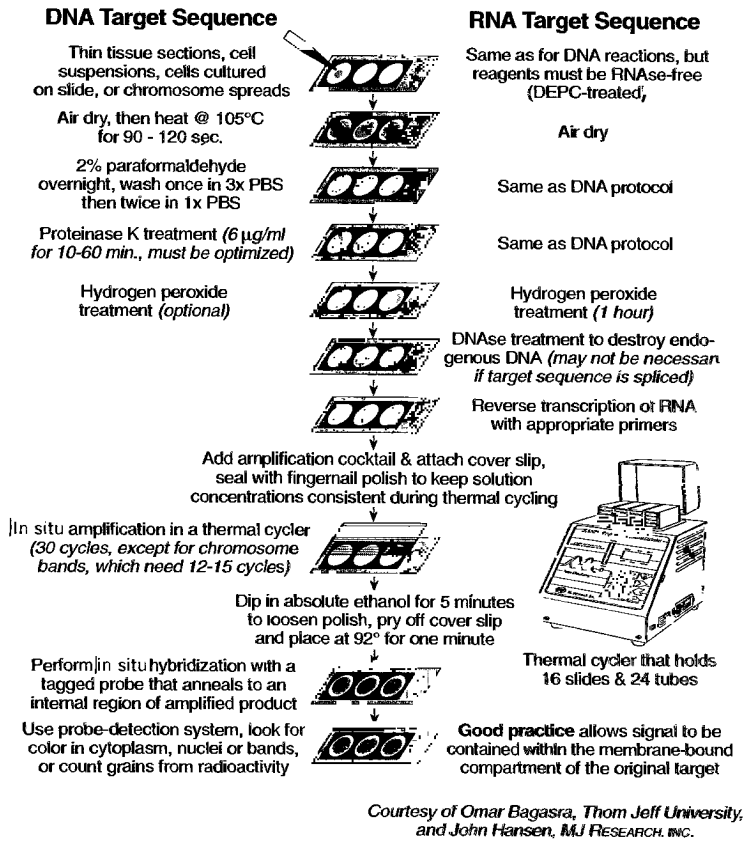

Figure 4.6. An overview of in situ PCR and hybridization.

2. Seal the slides with one of the following methods.

A. If using nail polish or adhesive to seal the slides, beginners often find it helpful to first pass a UHU Stic glue stick around the periphery of the glass coverslip (20×60 mm), such that a thin layer of glue (no globs) about 3 to 4 mm wide adheres to the perimeter. This will tack the coverslip in place while the nail polish is being applied (more experienced hands usually skip the glue).

For all practitioners, place the cover slip on top of the slide, or if using tissue sections, use a second, identical slide instead of a cover slip, as the second slide's Teflon coating will help form a more commodious reaction chamber. Carefully seal the edge of the cover slip to the slide (or the two

slides together) with clear nail polish or varnish (see "Discussions," page 54). Let the nail polish dry for 1 min; then place the slide on a heat block for 90 s at 92°C, to further cure the adhesive.

B. If using gene codes, follow the manufacturer's instructions for adhering the cone to the slide. Place the designated quantity of reaction cocktail into the chamber, and seal with the adhesive film supplied. We have sometimes found it useful to also place a weight on top of all the cones, as this will help keep the cones sealed during the thermal cycling.

C. If using slides that have cover slips with gaskets or a specialized thermal cycler with proprietary sealing apparatus, follow the manufacturer's instructions.

3. Place slides in an appropriate thermal cycler.
4. Run 30 cycles of the following amplification protocol:

Denaturation	94°C	30
Annealing (must be optimized)	45°C	1 min
Extension	72°C	1 min

As described earlier, these times and temperatures will likely require optimization for the specific primer pair employed in the reaction, as well as the specific thermal cycler being used. The annealing temperature especially should be optimized. These particular incubation parameters work well with SK-38 and SK-39 primers for the HIV-1 *gag* sequence, when amplified in an MJ Research PTC-100-16MS thermal cycler. Please note that there is no need for a prolonged initial denaturation in the cycling protocol, as denaturation was already achieved in the earlier steps.

6. After the thermal cycling is complete, open the slides. If the slides were sealed in nail polish, this is accomplished by dipping the slides in 100% EtOH for at least 5 min to loosen the nail polish (do not use acetone, which is a commercial nail polish remover, as this will damage the tissue irreversibly). Pry off the cover slip using a razor or other fine blade; the cover slip generally pops off quite easily. Scratch off any remaining nail polish on the outer edges of the slide so that fresh cover slips will lay evenly during the subsequent hybridization and detection steps. For other sealing technologies, follow the manufacturer's instructions.

7. Place the opened slides on a heat block at 92°C or back into the thermal cycler for 1 min. This treatment helps immobilize the intracellular signals.

8. Wash the slides with 2× SSC at room temperature for 5 min.

The amplification protocol is now complete and one can proceed to the labeling and hybridization procedures.

Optimized In Situ PCR with Self-Seal Technology An extremely useful innovation is Self-Seal reagent, which is an inert aqueous solution that is added to amplification cocktails to make slide sealing automatic. The reagent contains a mix of polymers, and upon initial denaturation, evaporation around the periphery of the cover glass forms a gummy, clotlike seal that limits subsequent evaporation during the cycling regime. To open the slides after cycling, one merely soaks the slide for a few minutes in an aqueous solution, and the cover glass slips off without any residue.

The manufacturer supplies this reagent as a 2× solution and has designed the product so that it works with a wide variety of in situ protocols, including in situ PCR, PRINS, and hybridizations. We have modified the recommended protocol of using 50% concentration by decreasing the amount of Self-Seal to 25% while adding 0.15% BSA and 0.1% collagen type 1. These latter two ingredients bolster the clotlike sealing on the Self-Seal, and we find that the annealing temperatures are largely unaffected by the reduced concentration of Self-Seal. Furthermore, we find this combination to give some of the best in situ PCR results we have seen.

Here is our optimized Self-Seal in situ PCR protocol (which can also be adapted to one-step RT PCR). **Note:** Do not use Teflon-coated slides with Self-Seal reagent. Rather use ordinary blank slides with proper saline surface treatment or special chambered slides sold by the Self-Seal manufacturer.

Self-Seal Reagent	25.0 μL
25 μM forward primer (SK 38 for HIV-1)	5.0 μL
25 μM reverse primer (SK 39 for HIV-1)	5.0 μL
10 mM each, 4dNTP	2.5 μL
10× PCR buffer	10.0 μL
10% BSA	1.5 μL
10% collagen type 1 (see note)	1.0 μL
taq polymerase (AmpliTaq 5 U/μL; see note)	2.0 μL
dd H_2O	40.5 μL
Total volume	*100 μL*

Note: Other thermostable polymerase enzymes have also been used quite successfully. However, one should always use the appropriate buffer for the specific polymerase, paying particularly close attention to the optimal $MgCl_2$ concentration.

1. Layer 50 μL of amplification solution over surface of a full slide, or 15 μL per chamber on a special three-chamber slide. Be careful to not touch the surface of the slide with the tip of the pipette.

2. Place a cover glass over the slide with the cocktail, the manufacturer recommends using a #2 cover glass. If there are air bubbles present in the chamber, allow the slide to rest 2 min; then using a blunt tool (like the eraser end of a pencil), gently press on the cover glass to coax the bubble to the periphery with out squishing out the cocktail. The bubble can be burst out the edge with a final (but gentle) press on the cover glass.

3. Place the slides into a thermal cycler that holds slides in a horizontal position. Begin cycling protocol; slides will seal automatically upon first denaturation step.

4. After thermal cycling, soak slides in dd H_2O for 5 min. The Self-Seal will then rehydrate and cover glasses will easily slip off slides. If one is particularly concerned about damage to the amplified tissue, soak the slides for 15 min and the cover glasses will fall away from slides when each slide is lifted from the water (the manufacturer also has another procedure for recovering the supernatant from a slide, if that is desired).

The amplification protocol is now complete, and you can proceed to the hybridization procedures.

In Situ PCR for One-step Reverse Transcription and PCR Use this protocol for one-step RT amplification, using either intron-spanning, RNA-specific primers (which allows both RNA and DNA messages to be amplified simultaneously) or tissue that has already received a DNAase treatment but has not undergone any reverse transcription reaction (this allows the use of a single buffering system for both reverse transcription and amplification).

This protocol is based on a new recombinant enzyme (r*Tth* thermostable polymerase), which can do both jobs at once. This enzyme has both reverse-transcriptase and DNA polymerase activity, and it is available in several variations from Perkin Elmer.

A typical cocktail for a single-step r*Tth* reaction follows. However, several of the r*Tth* enzymes come with their own special buffering systems, so use this protocol as a guide to adapt the manufactuer's recommendations.

100 μM primer 1	0.5 μL
100 μM primer 2	0.5 μL
10 mM each, 4dNTP	2.5 μL (each)
5× EZ buffer (see note)	20.0 μL
r*Tth*	4.0 μL
DEP × c = treated water	55.0 μL
25 mM $Mn(OAc)_2$	10.0 μL
Total volume	*100 μL*

Note: 5× EZ buffer: 250 mM bicine, pH 8.2, 575 mM KOAc, 40% (v/v) glycerol.

This protocol can also be adapted for use with Self-Seal reagent. However, no matter the sealing technology, this reaction requires a slightly variant thermal profile in the cycling regime. Our laboratory uses the following profile, which begins with this reverse transcription cycle:

Reverse transcription	60°C	30 min
Regular cycling (30 cycles)	94°C	30 s
	45°C	1 min
	72°C	1 min

Follow either of the procedures for slide loading, sealing, and handling recommended by either of the protocols described above.

Discussions

Attaching Cover Glasses with Nail Polish Be certain to carefully apply the glue and polish around the entire periphery of the cover glass or the edges of a dual slide, because the polish must completely seal the cover slide assembly to form a small reaction chamber that can contain the water vapor during thermal cycling. For effective sealing, do not use colored polish or any nail polish that is especially runny; our laboratory uses Wet & Wild Clear nail polish, though it is the viscosity, not the brand, that is critical. Use of the UHU glue stick

is not critical, rather, it assists beginners in keeping the cover glass in place while applying the nail polish; often the cover glass likes to slide around during sealing.

Proper sealing is exceedingly important, for this keeps reaction concentrations consistent throughout the thermal cycling procedure, and these concentrations are crucial to proper amplification. However, be certain to apply the nail polish carefully so that none of the polish gets into the actual chamber where the cells or tissues reside. If any nail polish does enter the chamber, discard that slide, for the results will be questionable. Please bear in mind that the painting of nail polish is truly a *learned* skill; therefore, it is strongly recommended that researchers practice this procedure several times with mock slides before attempting an experiment.

In the case of thicker tissue sections (>10 μL), it is better to use another identical, Teflon-coated slide for the cover instead of a cover glass. Apply the amplification cocktail (20 to 40 μL) to the appropriate well of the blank slide, place an inverted tissue-containing slide atop the blank slide, and seal the edges as described. Invert the slide once again so that the tissue-containing slide is on the bottom.

Alternatives for Sealing Slides and Attaching Tissues Other groups have reported different methods for creating vapor-tight reaction chambers. For example, Staskus (Ashley Haase's laboratory, University of Minnesota) has reported using a specialized thermal cycler that has four chambers in which pools of mineral oil 2.5 mm deep can be created. She reports that she uses silanated slides without Teflon coatings, and after preparing thin tissue sections, she layers a small amount of amplification cocktail on top of the tissue. Staskus then places a cover glass over the tissue section and wicks out the excess cocktail with a paper towel, so that the cover glass adheres tightly to the slide. Then the slides are carefully slipped into the pools of mineral oil and gently tamped down so that the slide assembly is in close contact with the cycler and the slide is fully immersed in oil. After cycling, the slides are removed and placed on a paper towel, which absorbs the excess oil. The slides are then washed in two baths of chloroform. Finally, the slides are immersed in PBS, and the cover slip is floated off. Staskus reports that this method has proven effective.

Gene Cones (Gene Tec Corp.; Durham, N.C.) eliminate the need for attaching cover slips. These small plastic chambers serve in lieu of cover slips by adhering to the slides with an adhesive, such that circular chamber

of 15 mm diameter is formed on the slide. Fluids can be added through an opening in the top, which is then sealed with an adhesive tape.

We have experimented with the Gene Cones, and we know they can prove useful, especially for the analysis of cell suspensions. However, often the treatment of entire tissue sections is indicated, and many sections are too large or too irregular to fit under a small round cone. Furthermore, the chambers generally require 60 to 100 μL of cocktail, which is 3 to 12 times as much fluid as used under a cover slip; this may affect thermal characteristics. The cones also add substantial height to the slide such, so they will not fit in most in situ thermal cyclers. However, the Gene Cones can easily accommodate a hot start, and future models may solve some of the current difficulties. In our laboratory, we rarely use Gene Cones, preferring the cover glass methods described.

Finally, there are new technologies of cover slip (both glass and plastic) with special gaskets to seal the slide, to create reaction chambers much the same as those that are created with the Teflon-coated slides, cover slips, and nail polish. One example is Frame Seal (MJ Research). A number of investigators have reported success with this methodology; however, gasketed slides are an emerging technology, and further developments can be expected.

Hot Start Technique There is much debate as to whether a hot start helps improve the specificity and sensitivity of amplification reactions. The argument is rooted in the hypothesis that double-stranded DNA "breathes" at temperatures well below denaturing temperatures, such that significant stretches of DNA will periodically open then reanneal. If the temperature is low enough, it is possible for a primer to find a nearly complementary spot during a breathing episode. Because of low temperature, the primer might anneal to regions that are not exactly complementary, and then the polymerase enzyme will partly extend the primer reading the DNA template. Unfortunately, the extended region would now be a perfect match to the wrong segment of DNA, and this extended primer could now serve as a fully effective false primer during subsequent amplification cycles. This could lead to some degree of unwanted amplification of regions of DNA other than the region targeted (though false primers are less likely to cause geometric amplification of the unwanted regions, as with dual-primed PCR of the targeted areas).

Those who advocate a hot start say that this unwanted annealing can be avoided if somehow the critical mix of DNA, primers, and polymerase

at low temperatures can be avoided during setup, such that these components first intermingle only at temperatures where false priming cannot occur no matter how much the DNA breathes. Thus hot starts generally call for adding the polymerase enzyme at the very end, after the reaction vessel and mix have been brought to high temperature (generally higher than 60° to 70°C).

As a matter of practicality, we find that the hot start adds little advantage and adds technical difficulty to the practice of the in situ technique. However, we are generally using carefully designed, highly optimized primers. There is no doubt in any intellectual camp that better primer design leads to less false priming, so our solution to nonspecific amplification is to design better primers rather than rely on the crutch of a hot start (though we generally run across false priming only in solution-based reactions analyzed on a gel, for the reasons described later).

Even if there is some degree of nonspecific amplification in the in situ reactions, that is not critically important. The type of in situ PCR described here, namely in situ amplification followed by a separate hybridization, relies primarily on the hybridization step for specificity not on PCR. Even if there are some nonspecific amplified messages within the amplified tissue, the hybridization probe will not pick them up; thus they will not show up in the final data.

Last but not least, it is our hypothesis that the 105°C heat treatment in the first stage of this protocol makes the hot start debate moot in this application. The breathing of the DNA which is thought to lead to false priming depends largely on specific enzymes known as topoisomerases and gyrases, which flex the DNA for specific transcriptional purposes. However, the heat treatment at 105°C in the beginning of the protocol deactivates these enzymes, so they are unable to flex the DNA to cause the circumstance where false priming might occur in the first place.

But we realize that some investigators are comfortable with the hot starts and would prefer to use them. Fortunately, there are several new developments among reagents to facilitate the hot start technique without adding the additional chore of having to add enzyme to preheated slides. The first such innovation to be introduced was a monoclonal antibody that blocks *taq* polymerase activity during the reaction setup. Upon the initial denaturation, the antibody is inactivated, so the overall results should be the same as with a hot start. This antibody is called TaqStart (Clontech Laboratories, Inc.; San Francisco). We have used this antibody successfully in our in situ protocols (to ascertain whether it interferes).

The second alternative is a new *taq* polymerase enzyme called AmpliTaq Gold (Perkin Elmer). This enzyme does not activate until the first denaturation step, so it too can mimic a hot start without any additional effort. Several colleagues have reported that this enzyme works better than the enzyme–antibody complex described above (at least in solution-based reactions), but this is a new product and we have no experience with it in situ. The one disadvantage is that Perkin Elmer recommends an initial denaturation of 10 to 12 min to activate the enzyme; this could conceivably compromise the morphology of the tissue as well as the activity of the *taq* polymerase enzyme.

Last but not least is the old manual hot start alternative, which has now been considerably eased by the introduction of Self-Seal reagent. Simply preheat a slide-based thermal cycler to 70°C, preheat the slides without cover glass or reaction cocktail to the same temperature, pipette on the proper aliquot of Self-Seal–amplification cocktail, apply the cover glass, and slip slide assembly into the thermal cycler. After the cycler is loaded with all slides, commence the PCR run, making certain that the cycler goes directly to the denaturation temperature. This will achieve the desired hot start.

Thermal Cyclers Various technologies of thermal cycler will work in this application; however, some instruments work much better than others. We began our investigations years ago with a standard, block-type thermal cycler that normally holds sixty 0.5-mL tubes but that was adapted with aluminum foil, paper towels, and a weight to hold 4 to 6 slides (Bagasra 1993). We have also used dedicated thermal cyclers that are specifically designed to hold 10, 12, and 16 slides; and our current instrument is a DNA Engine (MJ Research) with a dual independent block that holds 32 slides. We understand that other labs have used stirred-air, oven-type thermal cyclers quite successfully; however, these machines can be slow in operation. Thermal cyclers dedicated to glass slides are now available from a number of vendors, and some designs of thermal cycler incorporate humidification chambers that can assist in performing the subsequent hybridization steps. However, the humidified instruments do not eliminate the need for sealing slides during thermal cycling; rather, humidification is useful during steady-state incubations, as with hybridizations or reverse transcription reactions. As new products become available, look for thermal accuracy and uniformity, simplicity of

design, and ease of use, but do not underestimate the need or difficulty for keeping the slides sealed throughout a thermal cycling regime.

We suggest that you follow the manufacturer's instructions on the use of your own thermal cycler, bearing in mind the following points:

1. Glass slides do not easily make good thermal contact with the surfaces on which they rest. Therefore, if you are using a thermal cycler that relies solely on conduction of heat through the base of the slide (like several platformlike thermal cyclers), you should use a weight to press down the slides and/or a thin layer of mineral oil to fill in the interstices to help thermal conduction. If you are using mineral oil, make certain that the oil is well smeared over the glass surface so that the slide is not merely floating on air bubbles beneath it. Be sure to insulate the top of the slides in some manner.
2. The top surfaces of slides lose heat quite rapidly through radiation and convection; therefore, superior thermal cyclers envelope the slide in some manner. If the thermal cycler being used does not have this characteristic, make certain the slides are insulated in some manner.
3. Good thermal uniformity is imperative for good results; poor uniformity or irregular thermal change can result in cracked slides, uneven amplification, or completely failed reactions. Furthermore, keep in mind the critical nature of annealing temperatures within situ PCR; the reaction is simply not as robust as solution-based PCR. So quality instrumentation is imperative and we strongly recommend acquisition of any instrument that is dedicated to slides.
4. If there is no other alternative, you can adapt a thermal cycler that normally holds plastic tubes. Press a *single* layer of thick aluminum foil over the block, place the slides on top of the foil (usually four to six will fit), cover the slides with a 3- to 6-cm layer of paper towels for insulation; and use a weight of about 1 kg to press down the towels onto the slides (you must use the nail polish technique for sealing the slides with this apparatus). It was with such a device that this protocol was originally developed.
5. There are two brands of high quality thermal cycler dedicated to in situ that we have used successfully: 2 models made by MJ Research and one model by Perkin Elmer. Both companies offer novel slide-sealing technologies, and the top-of-the-line MJ Research ma-

chine can even double as a humidified incubator. A number of other brands of dedicated thermal cyclers are available as well; however, some of these have not proven useful in our hands. We are certain that there are other quality in situ instruments available; we just have not had the opportunity to try them yet.

Direct Incorporation of Nonradioactive Labeled Nucleotides Several nonradioactive labeled nucleotides are available from various sources (eg, dCTP-biotin and digoxin 11-dUTP). These nucleotides can be used to directly label amplification products, and then the proper secondary agents and chromogens can be used to detect the directly labeled in situ amplification products. However, in our opinion, as well as in the opinion of many other laboratory groups, the necessary specificity is achieved only by conducting amplification followed by subsequent in situ hybridization. In the direct labeling protocols, nonspecific incorporation can be significant; and even if this incorporation is minor, it still leads to false-positive signals similar to nonspecific bands in gel electrophoresis following solution-based DNA- or RT-amplification. Therefore, we strongly discourage the direct incorporation of labeled nucleotides as part of an in situ amplification protocol.

The only exception to this recommendation is when you are screening a large number of primer pairs for optimization of a specific assay; then direct incorporation may be useful. To perform such screenings, to the amplification cocktails described earlier, add the following: 4.3 μM labeled nucleotide (either 14-biotin dCTP, 14-biotin dATP, or 11-digoxigenin dUTP) along with unlabeled nucleotide to achieve a 0.14 mM final concentration.

CHAPTER 5

SPECIAL APPLICATION OF IN SITU AMPLIFICATION

ELECTRON MICROSCOPY

Both our group at Thomas Jefferson and another group at Uppsala University in Sweden have been developing techniques to observe the results of in situ amplification under electron microscopy (EM). The procedure both used is a simple modification of the immunogold EM technique.

First, the in situ amplification is carried out in solution rather than with the cells or tissue adhered to a glass slide. The cells or tissue are fixed in 4% paraformaldehyde for 4 h, washed in PBS buffer, and then treated with proteinase K. As before, the cells are observed under an optical microscope for the development of the peppery dots, as described earlier. Then amplification of DNA or RNA is carried out, as described earlier, in a cell suspension rather than fixed on a slide.

To check the validity of the results, a small portion of the cells is withdrawn and placed on a slide and in situ hybridization is performed with the specific biotinylated probe as well as an unrelated biotinylated probe as a negative control. The color is developed with streptavidin–peroxidase or streptavidin–alkaline phosphatase. If the color develops with the specific probe and the nonspecific control is negative, then immunolabeling can be performed with the remaining cells.

For this purpose, cells are hybridized with biotinylated probe(s) and then labeled with streptavidin–immunogold conjugates. After 1 h of incubation at 37°C, unbound conjugates are washed extensively and the cells are pelleted in a centrifuge. The cell pellet now can be incubated in freshly prepared 2.5% glutaraldehyde solution and processed for EM work.

EM grids can also be used as the substrate for the cells or tissues. Place the grid between two slides and perform the in situ amplification steps as described above. Then perform the labeling with the biotin–streptavidin–immunogold steps.

In our hands, we find the harsh treatment inflicted by repeated denaturation tends to destroy the internal organelles of cells. However, clear signals can be detected in the perinuclear areas of the cells.

IN SITU PCR AND IMMUNOHISTOCHEMISTRY

In situ PCR and immunohistochemistry can be performed consecutively on the same cells in a tissue section or mounted cell suspension. Pathologists and cytologists have tabulated specific surface and intracellular antigens that can withstand 10% formalin fixation and other routine histopathology procedures and still bind specific monoclonal antibodies. If immunohistochemical panels incorporating these specific antigen–antibody pairs are to be used, then routinely prepared, formalin-fixed paraffin sections can be used for in situ PCR and later for immunologic assays of cellular antigens. The resistant antigens include many of those used in routine diagnostic immunohistochemistry and pathology, including those listed below. In these cases, the tissue is prepared for in situ amplification as described earlier, including the 2% paraformaldehyde fixation. After color development of the hybridization probe, an antibody labeled with FITC or rhodamine is applied to observe potential colocalizations of signals.

However, if you want to detect the light chains of immunoglobulins (or any other labile antigen listed below) by immunohistochemical means after in situ PCR, special treatment is needed. Cells or frozen sections of tissue (which are already placed on slides) are prefixed in 100% methanol for 10 min. The slides are then washed in PBS and are incubated with unlabeled, FITC-labeled, or rhodamine-labeled antibodies for 1 h. The slides are washed again several times in PBS, and then they are

fixed with 4% paraformaldehyde for 2 h. In situ PCR is now conducted as described, starting with the 105°C heat treatment but skipping the overnight 2% paraformaldehyde fixation. If unlabeled probes were used initially, then appropriate incubations with secondary antibodies conjugated with FITC or rhodamine are carried out after amplification.

Some investigators have reported success with labile antigens using Permiofix, a new fixative (Ortho Diagnostics, Rarifan, N.J.) that is less destructive to cell surface antigens than formalin. However, according to the manufacturer, Permiofix is an experimental fixative that has generally been supplied to specific flow cytometry laboratories only. Therefore, you may have to lobby aggressively if you wish to try some.

The immunohistochemical procedures themselves are carried out by standard methods, with just the minor adaptations described above. If using immunohistochemistry with in situ PCR, it is helpful to use a combination of uv and visible light detection systems so that the two signals can be viewed in an alternate fashion under these two types of illumination. Alternatively, amplicons can be hybridized with FITC-labeled probe and antibody with rhodamine, and slides can be viewed under a dual-color filter.

Cellular Antigens Resistant to Formalin Fixation

The following antigens are resistant to formalin fixation: all variants of keratin, vimentin, and desmin; muscle-specific and prostate-specific antigens; prostate alkaline phosphatases; various lymphoma and leukemia markers, including CD3, CD21, CD30(T_1), LCA, and L26; germ cell markers; fetal tumor markers; α-fetoprotein; placental alkaline phosphatase; all the neuroendocrine antigens; many hormones, including thyroid, pituitary, many of the hypothalamic-releasing factors, estrogen, and progesterone; some antigens used for breast cancer detection; many central nervous system markers; GFAP; NSE; S-100; RCA-1; VWF; and factor VIII.

Labile Cellular Antigens Not Resistant to Formalin Fixation

The following antigens are not resistant to formalin fixation; κ and λ light chains of immunoglobulins, CNPase for oligo dendrocyte detection, and numerous other cell surface antigens.

MULTIPLE SIGNALS, MULTIPLE LABELS IN INDIVIDUAL CELLS

DNA, mRNA, and protein can all be detected simultaneously in individual cells. As described earlier, it is possible to label proteins by rhodamine-labeled antibodies, and then perform both RNA and DNA PCR in situ (if RNA-specific primers, as described on pages 9–11, is used along with a DNA primer pair). Subsequently, products can be labeled with different kinds of probes, resulting in different colors of signal. For example, proteins can have a rhodamine or phycoerythricine probe, mRNA can show a FITC signal (or one of 20 different fluorochromes that are available), and DNA can be labeled with a biotin–streptavidin–peroxidase probe. Each will show a different signal within an individual cell.

RECOVERY OF AMPLICONS FOR SEQUENCING AND CLONING

Sometimes it is desirable to recover samples of the amplified products from in situ reactions. For example, perhaps a sample is needed to sequence amplicons so that various alleles of a gene, or even various expressions of a gene, can be distinguished. Perhaps an investigator wishes to clone the gene in question to obtain larger quantities for future study.

One of the most exciting applications is in the field of developmental biology: amplification products can help determine what proteins are involved in the processes of cellular differentiation and organogenesis. For example, sections of embryos can be taken at various stages of development, and specific or oligo d(T) primers can be used to identify the gene expression that is occurring in particular index cells. If you reserve and store the supernatant after amplification, then you can go back and reamplify the gene of interest once the specific phase of differentiation has been identified in the index cells following hybridization of the whole tissue. In this circumstance, you might even use a micromanipulator to recover the specific cells to study gene expression more closely.

By simply reserving the amplification cocktail following the thermal cycling procedure, recovery of the amplicon can usually be achieved. It has been our experience that there is usually a small amount of leakage out from the cells into the amplification cocktail during amplification; but

with proper proteinase K digestions, there is little or no leakage into other cells in the tissue sample. We have collected abundant data on this matter, in particular by running samples of the supernatant in electrophoretic gels following amplification. We have found that amplified products can usually be detected by Southern blotting but not by ethidium bromide staining, as the signal is weak. However, this product can usually be reamplified in a subsequent solution-based reaction using the same primers as the in situ reaction (or a nested primer pair), and this provides sufficient quantities of the amplicon for subsequent cloning or analysis.

We hypothesize that the leakage of signal in the original in situ reaction tends to occur late in thermal cycling, as the signal recovered from the supernatant is almost invariably quite weak (at least when the proteinase K digestion was properly optimized). In the latter cycles of the PCR procedure, the geometric nature of the amplification makes the concentration of the amplified product high at the original locus of the target, and it is reasonable to assume that some small fraction of the amplified signal could drift away from this locus and diffuse out of the cell. This would be particularly true for those positive cells whose nuclear or cytoplasmic membranes were sliced open by the blade of the microtome, such that a primary containment vessel of the signal was violated. However, if this diffusion were occurring in significant quantity or early in the amplification process, we would expect to see strong signals in the supernatant—manifested both in the agarose gel and in intercytoplasmic staining following hybridization—as subsequent amplification would have occurred in the supernatant. In fact, we do not see strong signals, except in those circumstances where there was excessive digestion with proteinase K.

Finally, we have encountered no evidence to indicate that leaked signals enter into other cells to deliver false-positives upon subsequent hybridization, as we believe this would require fairly large membrane pores. Rather, we hypothesize that during the optimized proteinase K digestion, there is only partial digestion of certain transmembrane proteins, which results in the formation of semipermeable pores. These pores selectively allow positively charged molecules to pass, but they tend to block negatively charged molecules. Therefore, the *taq* enzyme and single-stranded small primers can enter the cell, but double-stranded amplicons (which are highly negatively charged) cannot readily pass. Therefore, false signals do not enter the cells.

To recover the supernatant, special attention must be paid to the choice of sealing technology for the slide as well as the procedure for opening the slide following amplification. The sealing technology that gives the easiest recovery is the Gene Cone, but this technology has other limitations, as noted earlier. Recovery can also be made with gasketed cover slips, but the ease of recovery varies with the particular design of the cover slip. Recovery can also be achieved with slides sealed with nail polish or Self Seal reagent, and this is usually achieved by slitting a corner of the seal with a razor blade, and then sucking out supernatant with a capillary tube, a small pipette tip, or a 1-mL tuberculin syringe with a 26-gauge needle. Try to work over a sheet of parafilm so if some of the supernatant slides out, it can still be recovered.

In reamplifying the signal, the same primer pair can be used as in the original PCR amplification. However, it is common practice in molecular biology to use nested primers—ones that are slightly inboard of the original primer pair—for reamplification reactions, as these primers generally anneal more readily to amplicons and give better results.

IN SITU PCR AND GENE THERAPY REGIMES

One of the biggest hurdles faced by gene therapy investigators is the lack of any convenient means to assess the degree to which a vector has inserted a new gene into the genomes of a target tissue as well as to assess the degree and circumstance in which the gene's product is being expressed. In situ PCR represents a unique tool that can provide these much-sought data relatively easily, particularly when the technique is multiplexed to assay several nucleotide targets at once and it is combined with immunohistochemistry to detect the type of cell transfected or the type of cell in which the transgene is being expressed.

Following transfection or transduction, investigators generally want to know two things: (1) did the transgene insert itself into the correct cells in sufficient quantity and with the proper copy number, and (2) is the transgene exhibiting the controlled expression that it was designed to do. The difficulties in making these assessments lie in the fact that vectors are usually designed to insert only one or two copies of a sequence into the genome of any particular cell, and the subsequent low abundance nucleotide targets are difficult to detect. If solution-based PCR or Southern blotting is used, you can tell if a gene is present in the target tissue; but it is difficult to glean quantitative data on the percentage of cells infected or

the type of cells or the number of copies inserted. If you use immunohistochemical techniques to detect expression, high levels of expression are required to detect the gene product. Abundant expression is not necessarily available or desired in many instances, as this may interfere with the normal functioning of the cell.

In situ PCR can solve these data-acquisition problems quite readily. For example, our laboratory has been working on a gene therapy treatment for those already infected with HIV-1. First CD34 cells (ie, stem cells from bone marrow or cord blood) are transfected ex vivo with a gene that is designed not to express itself until it sees the protein encoded by the *rev* gene of HIV-1. If this protein appears in the cell, the transgene is switched on and expresses an intracellular antibody that binds this protein, shutting down HIV-1's ability to assemble its genome. These transfected CD34 cells are injected in vivo, where they differentiate to produce various lymphocytes and restock a compromised immune system.

A similar strategy has proven to be quite effective in tissue culture, and it will be undergoing human trials at Thomas Jefferson University. To develop this system and monitor its progress, in situ PCR has been and continues to be absolutely critical. First, it has allowed us to develop a low expression vector whose integration can be assayed directly in tissue culture. Second, by combining DNA and RNA amplification by using RNA-specific primers (detailed on pages 9–18, 48–50), we are able to monitor the presence of the dormant gene in the *transfected* cells that are not non-expressing HIV-1 (this is a circumstance where the transgene should not be expressing at all). By seeking the *gag* sequence from HIV-1 simultaneously, we are able to tell if the gene is expressing properly in the infected cells when it should be. By combining these techniques with immunohistochemistry, we are able to specifically identify the cell types carrying the gene and the cell types that are infected with HIV-1 as well as the percentages these fractions represent of the total population in vivo. Finally, by amplifying a *tat–rav* multiply spliced RNA sequence of HIV-1 simultaneously with a DNA sequence of the transgene, we are able to tell if our strategy for shutting down HIV-1 continues to function in the longer term. These sort of data will prove to be invaluable for our investigation, as well as for others who are seeking similar information.

Similar strategies can be employed to develop and monitor the progress of other gene therapy regimes, particularly those for treating other infectious or genetic diseases as well as gene therapy strategies for fighting tumors with suicide construct transgenes. We have found in situ

PCR to be much more elucidating than Southern blots, Northern blots, solution-based PCR, branched-chain PCR, and advanced tissue-culture plaque assays. However, to the best of our knowledge, no other group is currently using in situ PCR with gene therapy experiments. We strongly encourage these researchers to investigate the possibilities.

IN SITU PCR ON CHROMOSOMES

Discussion of Uses

Often it is helpful to identify the locus of a gene long before much is really known about its sequence. By conducting in situ PCR on chromosome spreads, it is possible to determine the locus knowing as little as 80 to 100 bp of the sequence. No longer is it necessary to clone thousands of base pairs to create a probe; rather, synthesized probes as short as 40 bp can manifest a strong signal clearly discernible in ordinary bright-field or fluorescent microscopy. Furthermore, the hybridization procedure is much less tedious than with fluorescence in situ hybridization (FISH), as the genetic signal is so much stronger. This methodology is not only useful in mapping but is also proven helpful in the study of retroviral diseases as well as in the development of gene therapies, for it is possible to assay the incorporation of sequences into the genomic DNA.

For example, our laboratory has successfully amplified an HIV-1 sequence on chromosomal bands prepared from SUP-T1-infected cell lines. We are also using this technique to assay peripheral blood mononuclear cells from HIV-1-infected individuals. We have simply modified a typical chromosomal banding procedure so that it can incorporate in situ amplification, which mainly involves creating an "artificial" membrane over the chromosomes to contain the amplified signal. The process is similar to the in situ amplification procedure described earlier, except for the following modifications: (1) chromosomal spreads are made onto specially prepared slides, (2) the naked chromosomes are covered with ghost membranes from the original culture so that the signal will remain localized, (3) the heat fixation step is eliminated, and (4) fewer cycles (10 to 12) of amplification are used so that the signal will not become so strong and diffused that resolution of the original locus of the gene becomes difficult.

Typically, the process starts with a primary culture or a tissue culture with sufficient cell mass. At this time, a portion of the culture is reserved, and these cells will later be swollen and burst with a hypotonic solution, which is the source of the ghost membranes. The other portion of the culture will serve as the source of the chromosomes, and these cells usually require stimulation with a mitogen such a phytohemagglutinin (PHA). They are then arrested at mitotic metaphase by blocking spindle formation with a spindle inhibitor such as vinblastine (velban), colchicine (DAMC), or colcemid. The cells are next dropped onto specially prepared slides to make chromosome spreads, and an aliquot of supernatant from the membrane culture is dried atop the spreads to create an artificial membrane that will hold the amplified signal at its original locus on the chromosome.

Now the prepared slides are fixed in a mixture of methanol and glacial acetic acid (GAA) in a 3:1 ratio. The methanol serves to harden the chromosomes, helping to preserve their morphology, while the GAA has a softening effect, helping to open the histones for molecular manipulation of the nucleotides.

In situ PCR is performed, with primers of 18 to 22 bp. The target for amplification should be 80 to 500 bp, and subsequent hybridization should be made with a tagged probe of 40 to 100 bp that is separated from both primer sequences by at least 2 to 3 bp. The chromosomes are then counterstained to develop the banding pattern, and analysis is performed under bright field, phase-contrast, or fluorescent microscopy. With good laboratory practice, it is possible to determine the physical location of the gene of interest or, for retroviral disease or retroviral vectors, the various loci of incorporation.

Protocol: In Situ PCR on Chromosome Spreads

Precautions Sterile technique must be practiced at all times during the culturing and prefixation parts of this procedure. Sterility is especially important in handling cell cultures, both to protect the investigator and to avoid introducing microbial contamination of the cell culture system. Such contamination is often the cause of test failure.

The human peripheral blood used in this procedure *may be infectious or hazardous* to the investigator. Proper handling and decontamination and disposal of waste material must be emphasized.

Initial Setup

Peripheral Blood

1. Divide cells into two portions
2. To two 25-mL culture flasks containing 5 mL RPMI media (see Chapter 8) add 0.5 mL of well-mixed whole blood. Rinse the pipet three or four times to expel all of the whole blood into the culture flasks (prepare three culture tubes for blood from newborns, to better ensure success).
3. With a 1-mL syringe, slowly add 0.1 mL phytohemagglutinin (PhA-C; see Chapter 8). Gently vortex or invert to ensure complete mixing.
4. Place the cultures in a CO_2 tissue–culture incubator. Loosen the cap to allow CO_2 penetration, if the culture flasks do not contain filter caps.
5. Incubate 66 h at 37°C with 5% CO_2 and 95% humidity.

Cultured Cells With culture cells, it is possible to synchronize the cell cycle so that subsequently large numbers of cells arrested at mitotic metaphase can be obtained. We can usually increase the number of cells arrested in mitosis to more than 50%, up from the more typical proportion of 1 to 25%. Cloned cell lines containing the gene of interest are generally most useful, but many cell lines will work, provided the cells grow exponentially in an appropriate medium, such as RPMI 1640 in 10% FBS.

1. Divide culture into two 150-mL flasks or Cell Factories, each with more than 100 mL of medium. Incubate for 48 h at 37°C in a humidified incubator.
2. To one flask only, add thymidine for a final concentration of 7.5 mM. Return the flask to the incubator; the length of this incubation should be equal to the combined periods of $G1+G2+M$ of the growth cycle of this specific cell line: typically, 9 to 12 h. At the end of this incubation, most of the cells in the flask will be arrested in the S phase. If the cells in the second flask require feeding, change the medium.
3. Wash the cells to remove the medium containing the thymidine and grow cells in fresh medium for 6 to 8 h more. At the end of this incubation, most cells will be exiting from the S phase.

4. Repeat Steps 2 and 3 to further synchronize the cells. Following 8 to 10 h of incubation without thymidine, most cells will enter mitosis. This can be confirmed by observation of the cells under phase-contrast microscopy. Mitosis can be arrested in mitotic metaphase by velban (described below).

Arresting Cells at Mitotic Metaphase

1. After incubation, remove the chromosome source cultures from the incubator and gently resuspend to ensure a homogeneous mixture.
2. Add 0.1 mL of working velban (see Chapter 8) to each tube or flask.
3. Mix each culture by gently swirling. Return them to the CO_2 incubator for 45 m. At this time, place hypotonic solution sufficient for the membrane-source cultures in the 37°C incubator to prewarm.

Harvesting

1. After 45 min in velban, centrifuge the culture tubes for 10 min at 800 *g*.
2. Aspirate and discard the supernatant leaving 0.25 to 0.50 mL of liquid on top of the packed cells.
3. Resuspend the cells by mixing on a vortex at the lowest setting.
4. *Slowly* add 5 to 10 mL of prewarmed hypotonic solution to each tube while vortexing.
5. Gently invert the tubes and place them in a 37°C water bath for 45 to 50 min.
6. Centrifuge for 15 min at 800 *g*.
7. Aspirate and *save* the supernatant, leaving 0.25 to 0.5 mL of liquid on the packed cells. The supernatant contains the ghost membranes, which will be used later to form an artificial membrane over the chromosome spreads.
8. Resuspend the cells in remaining supernatant by gently mixing with the vortex mixer at the lowest setting.
9. Using a Pasteur pipet with a rubber bulb while continuing to vor-

tex, *very slowly* add the fixative solution (see Chapter 8) to bring the total volume to 10 mL in each tube.

10. Allow the tubes to stand at room temperature for a minimum of 25 min.

11. Repeat Steps 6 through 9 for a total of three changes of fixative. However, there is no need to save subsequent supernatants from Step 7.

12. After the third fixation, spin at 800 g; aspirate the supernatant, leaving 0.10 to 0.20 mL on a layer of fixed white blood cells.

13. Add 0.10 to 1.0 mL of fresh fixative to the suspension; the amount depends on the density of the cell button.

Slide Preparation

1. Resuspend the button by bubbling with a fresh Pasteur pipet. Be careful not to draw any liquid into the wide portion of pipet; this would make recovery of the mitoses difficult.

2. Prepare single-well silanated slides, as described earlier. Dip the slides in DEPC distilled water. Freeze for 30 min before use.

3. Place the wet, prechilled slide onto the bench while dropping 3 to 5 drops of specimen 1.25 to 1.75 m (this may take practice) directly onto the slide. Allow the slides to air dry.

4. Add 2 to 3 drops of the supernatant saved in Step 7 of "Harvesting," which contains the ghost membranes. Allow the slides to air dry once again. An artificial membrane will develop over the chromosomes, which will hold the amplicons at their original loci.

5. Cure the slides on a hot plate at 59 to 60°C for 30 to 48 h.

6. In situ procedures are now begun, but do not heat fix at 105°C as in the standard protocol! Rather begin the in situ procedure with 2% paraformaldehyde treatment at room temperature for 1 to 2 h. Again at room temperature, treat with proteinase K (6 μg/mL) for 3 min only (do not overdigest). Inactivate the proteinase K by placing the slides on a 95°C heat block for 2 min. Wash in DEPC-treated water. Air dry.

7. Perform the rest of the in situ amplification as described with one modification: use only 10 to 12 cycles instead of 30.

IN SITU PCR ON PLANT TISSUES

Discussion of Uses

There has been a recent surge of interest in the assay of plant tissues through in situ PCR, largely as a consequence of transgenic research. Some researchers are striving to develop disease or insect resistance in plants, others are trying to better understand the complexities of gene expression in plants, and some truly pioneering investigators are trying to get plants to express encapsulated antigen proteins for human and animal diseases to create edible vaccines for a variety of mammalian scourges. The field of molecular plant research is really quite broad and exciting.

Compared to mammalian tissues, nucleic acids tend to be much more abundant in plant cells, but the signals being sought are often equally diffuse—whether they be RNA or DNA. Furthermore, there is often an abundance of nucleases in the plant tissue that rapidly degrades many messages, so traditionally sensitive assays, like Northern blots, are often difficult to conduct or they lead to false-negative results.

Unfortunately, the chitin in the cell walls of plants makes in situ PCR somewhat difficult to perform as well (other complex carbohydrates like lignin or pectin are involved with cell walls, of course, but chitin seems to be the really tough one). The chitin not only puts up a strong mechanical barrier against the diffusion of primers and enzymes into the interior of the cell but also tends to bind critical ions (such as magnesium) almost like a cation column. Thus the key to successful in situ PCR is the complete removal of the chitin through enzymatic digestion, releasing free protoplasts that can be spread onto a glass slide for assay. However, to keep the abundant nucleases from degrading the genetic signals, the protoplasts are kept alive throughout the chitin-removal procedure by adapting the digestion procedure of tried-and-true protocols for the preparation of cells for transgenic infection.

In fact, the plant cells should remain alive until the initial 105°C heat treatment at the beginning of the amplification procedure, at which time the nucleases are inactivated. The sought-after signals are then amplified and hybridized as with mammalian cells; and it is possible to readily detect single copies of the sequence in question. This is particularly helpful in transgenic work, because often only one or two copies of the gene of interest integrates into the host chromosomes. This technique can be used

to accurately assay the number of cells that successfully incorporated a transgenic cassette, or the genetic shot from a "gene gun."

In situ PCR with intact sections of plants is more difficult but still possible (with the exception of the fruiting bodies of plants, which are easier to amplify because they contain little chitin). We have been experimenting with frozen sections in particular, but the procedure is more difficult to conduct due to the need to remove the chitin without destroying the tissue structure. Unfortunately, a special cyrostat microtome apparatus is required, which we have had difficulty in obtaining. Thus our protocol is not quite ready for distribution. However, it is essentially the same as that described for protoplasts, except that thin sections (5 to 8 μm) are used, and we conduct the various rinses not with centrifugation and pelleting but with fresh baths of the various solutions.

Protocol: In Situ PCR on Protoplasts

Liberating Protoplasts with Enzyme Digestion

1. Harvest the leaves and sterilize the surfaces by immersion in a 50% ethanol solution for several seconds, followed by 10% bleach solution for 30 s. To rinse, immerse the leaves in a container of distilled water, pour off the water, and begin a series of three 2-min washes in distilled water.

2. To liberate the protoplasts, prepare a solution of mannitol and bring it to proper pH by adding NaOH or HCl. For tomatoes, a solution at 0.78 M and pH 7.0 works best; for tobacco, use 0.6 M and pH 5.4. Petri dishes are the best vessels for digestion: prepare about 20 mL of mannitol solution per 4-inch dish (one dish should be sufficient). Divide the prepared solution into two equal aliquots; one will be used unmodified and the other will be used to make an enzyme solution.

For an enzyme solution for use with tomatoes, add 46.8 mg Cellulysin, 15.6 mg. of macerase, and 12.5 mg of potassium dextran sulfate for each 10 mL of mannitol. For tobacco, use 37.5 mg Cellulysin, 12.5 mg macerase, and 12.5 mg potassium dextran sulfate for each 10 mL.

3. Using a razor blade, select tomato leaves about 5 cm or smaller. Make 1-mm slices at an angle without severing the midribs of the leaves. Float the sliced leaves in mannitol solution without enzyme until all the leaves are prepared; only the bottoms of the leaves should be in contact

with the mannitol. After covering the solution with leaf slices, gently withdraw the mannitol solution from the bottom of the petri dish with a syringe or pipette and replace with the enzyme solution. The leaves should again float on top of the solution.

4. Place the petri dish in a light-excluding container or wrap it in aluminum foil. Incubate at room temperature overnight, preferably in a slow-moving shaker.

Collecting Protoplasts and Rinsing away Enzyme

1. The following day, gently agitate the petri dishes to help liberate the protoplasts.

2. Pour the contents of the petri dishes through a MIRA cloth into a 50-mL plastic centrifuge tube. Pellet the protoplasts through low speed centrifugation (40 to 140 g), typically 5 min at 500 to 1000 rpm in a swinging-bucket rotor without braking.

3. Aspirate the enzyme solution and resuspend the cells in 1× PBS, filling the tube. Gently centrifuge the tube again.

4. Aspirate PBS solution, refill tube with fresh 1× PBS, and resuspend the pellet. Centrifuge once again.

5. Aspirate the PBS solution and resuspend the cells in 2 mL of 1× PBS. Place the tube in an ice bucket.

Counting Cells and Slide Mounting

1. Using a standard hemocytometer, count a sample of cells. Calculate the total number of cells in the suspension, and adjust the concentration to 5×10^5 cells/mL by diluting with PBS. However, the size of some plant cells is quite large; in these cases, dilute the solution to 1×10^5 cells/mL (or even further) so the cells will not crowd the slide surface.

2. Take silanated, Teflon-printed glass slides (or the equivalent), and add 10μL of the cell suspension to each of three wells using a P20 micropipette. Air dry the slide in a laminar flow hood.

3. As soon as slides are fairly dry, proceed with amplification or reverse transcription steps as you would with mammalian tissues, (described in Chapter 4). If this procedure is followed properly, the cells should remain living up to the 105°C heat treatment step, at which time the various nucleases are deactivated.

CHAPTER 6

HYBRIDIZATION REACTIONS

OVERVIEW

As the in situ amplification–hybridization procedure is both an outgrowth and an application of earlier in situ hybridization (ISH) techniques, we would like to highlight some of the pertinent aspects of the broader scope of ISH methodologies. We do this so the researcher can gain a better understanding of the various parameters of ISH and an appreciation of the relative advantages compared to alternative technologies, like Southern blotting or immunohistochemistry.

The ISH method involves the specific annealing of a labeled nucleic acid probe to complementary sequences within a fixed tissue (or within cells or chromosome spreads) mounted on a microscope slide or some other substrate. The nucleotide probe can be a synthetic oligo probe as short as 25 to 30 bp or a riboprobe many thousands of base pairs long; either can give extraordinary specificity if the annealing conditions, particularly temperature, are properly optimized.

For most of the nearly 30-year history of ISH, hybridization has been done to RNA targets to image the expression of various genes at the cellular level, as most ISH protocols need 20 to 100 copies of the target molecule per cell for the signal to be distinguished from background (for detection of new genes using new probes or tissues, often hundreds of

molecules are required). Most DNA targets and many virological targets simply do not occur in that high a titer, so other hybridization methodologies, including in situ PCR, have been developed to detect low abundance targets. These especially sensitive technologies also include fluorescence in situ hybridization (FISH), where fluorescently labeled riboprobes or oligo probes are annealed to chromosome spreads for "chromosome painting," or a visual form of gene mapping, and PRINS, a similar method that involves amplifying the target signal arithmetically through a single-primer procedure similar to thermal-cycle sequencing.

The goal of all these procedures is the ability to visualize—through a microscope, confocal, or image-analysis-based system—the signal of the original nucleotide target in its original locus in the specimen. Much qualitative and some quantitative information can be gleaned from these data, and substantial new discovery has resulted.

In terms of the methods of probe annealing and detection, all of the various ISH technologies are similar to the earlier Southern blot procedure, except that hybridization occurs in situ. The great power of ISH over Southern blots (or Northern blots, or dot blots) lies in its ability to detect the nucleic acid sequence within the intact cell, in its original context. It uniquely shows the signal superposed over the cellular and subcellular details of the specimen, something blotting (and gel electrophoresis) procedures are wholly unable to do.

However, the Achilles' heel of in situ hybridization lies in the fact that the nucleic acids are bound inside a complex matrix of cellular structures, so that the target sequences are not readily accessible to the labeled probes. Thus hybridization can be difficult to carry off. This can decrease the overall number of probe–target hybrids formed when compared to nucleic acid targets immobilized on a nylon–nitrocellulose membrane (as in Southern and Northern blots). Furthermore, the investigator must optimize some of the parameters of ISH empirically by trying small modifications and seeing what works best.

In the early days, radioisotopes were the only effective labels available for nucleic acid probes; therefore, autoradiography was the only means of detecting hybridization sequences. Unfortunately, this often involved long exposures to detect the signal (a week, even a month, was common) as well as the inconvenience, hazards, and regulatory hassle involved with radioactivity. But detection technology has advanced markedly in the past few years, and there is now a cornucopia of nonradioactively labeled probes that work quite effectively with ISH, especially if the signal

has first been amplified through in situ PCR. Thus ISH has become more sophisticated and available to a wider variety of laboratories in both research and clinical settings.

However, a universal, reproducible "gold-standard" procedure to fit all situations has never been developed with in situ hybridization. Therefore, the specific needs of the diagnostic or research goals must be carefully considered before choosing this methodology. If in situ PCR or ISH are decided on, then more thought is needed in designing a protocol that fits, the specific application (eg, what tissue preparation to use, what nucleotide sequence to target, and what method of detection to use).

RELATIVE MERITS VERSUS OTHER TECHNIQUES

In analyzing any laboratory technique, an investigator must bear in mind the type of signal being detected. Immunohistochemical techniques, for example, can localize a protein within a cell or on the cell surface and, therefore, identify specific aspects of gene expression. However, these assays cannot yield useful information on posttranslational processing of the gene product or differentiate between the uptake–storage areas for the protein and the site of synthesis. In addition, hundreds of molecules of the protein are needed to identify expression through immunohistochemical methods. But the technique is relatively easy to perform and useful in many circumstances.

Similarly, mRNA extraction methods that rely on the isolation of nucleic acids from cells (ie, membrane–hybridization assays) can give useful information relatively easily, but they cannot distinguish between strong signals expressed in a few cells from weak signals expressed in many cells. In fact, there is no useful distributional information in these assays whatsoever, rather there is only an average measurement of the target present in what might have been a mixed population of cells.

Quite simply, ISH can yield much more information than either immunohistochemistry or filter assays, but it is a tougher procedure to perform. Its chief limitation revolves around its relative insensitivity (at least when compared with in situ PCR) when weak signals are being sought. The best that can possibly be achieved with ISH alone is perhaps detection of as few as 20 copies of mRNA per cell; however, that degree of sensitivity is limited to a few highly specialized laboratories and to a few specific genes. More realistically, detection of > 100 copies/cell would

be an achievable goal for a laboratory not specialized in ISH. To reliably detect a single copy of a gene (DNA) or to detect low levels of gene expression (mRNA), it is necessary to first amplify the nucleotide sequences in situ with PCR, RT-PCR, or ligase chain reaction (LCR), and then use ISH to detect the amplicons.

RADIOACTIVE PROBES VERSUS NONRADIOACTIVE DETECTION

Many ISH protocols employ ^{3}H or ^{35}S-labeled probes, followed by autoradiographic detection. Although this method can be sensitive and ^{3}H-labeled probes generate high resolution autoradiographic signals, use of these radiolabeled probes is time-consuming and technically difficult. Other, higher emitting radioisotopes can be used as well, but these give higher nonspecific background.

Nonisotopic methods for ISH offer the advantages of probe stability, sensitivity, spacial resolution, and time savings. The nonisotopic adaptations are generally simpler and faster than autoradiography, and the sensitivity of nonradioactive methods has increased over the years as the parameters influencing the hybridization efficiency and signal specificity have become better understood and better optimized. Some recently developed benefits include faster color development times, chemically stable probes with no special disposal requirements, and detection systems that allow the analysis of several probes simultaneously.

THE EMPIRICAL ARTS OF ISH

Because the detection of nucleic acids in situ is complicated by the fact that the signals are located within the complex matrix of a cell, it is important to optimize empirically the various experimental parameters to the specific application. This is the art of the technique, and the many protocols employed by various laboratories are illustrative of the styles of the various masters. There is simply no single right way to conduct ISH (though there are many wrong ways).

Rather each investigator should consider the relative needs for specificity, sensitivity, signal resolution, idiot proofing, and speed of operation when tailoring a protocol to individual needs. Systematic analysis of suc-

cesses and failures in our own laboratory has determined the following parameters to be ones that require special attention:

- Method of sample preservation (ie, fixation).
- Hybridization temperature (very important).
- Probe size, concentration, and design.
- Ionic concentration in hybridization solution.
- Method of detection (we always use non-rad methods).

Be sure to pay careful attention to each of these details when designing a protocol.

HYBRIDIZATION AFTER IN SITU PCR AMPLIFICATION

As hybridization after PCR amplification is a unique circumstance (and this monograph is dedicated to this technique) the remainder of this chapter is devoted exclusively to hybridization following PCR.

Probes

The choice of probe type depends on the intended target. When there are abundant copies of a target, as is the case after most PCR reactions, a relatively insensitive probe or detection system can be used without compromising the results. When there are low levels of target (as is usually the case with simple ISH without PCR), long riboprobes are usually used as they are the most sensitive.

Furthermore, in solution, the most rapid hybridization occurs with long probes at high concentrations. Because of problems with probe penetration when using intact cells or tissues, shorter probes give the more rapid hybridization in situ, as they can more easily gain access to the targets. We typically use synthetic oligo probes of 40 to 80 bp in length.

Penetration of the probe into the cells can be further compromised by molecules used to label or tag the probe for detection. These molecules can increase the rigidity of the probe, increase the steric hindrance, and adversely affect the charge density and the distribution of the probe. The proper probe concentration must also be determined to give the best signal to background ratio. We usually use 20 pM of labeled probe.

For most DNA targets, we use oligo probes, but we have also used probes that we have PCR amplified and labeled from genomic DNA templates. Also useful are cDNA probes that have been cloned from a vector or isolated from that vector, especially if you are trying to discover the locus on a chromosome for that expressed sequence. For RNA targets, we prefer oligo probes again, but you can also use probes generated by cDNA sequences that have been cloned into appropriate vectors (but do not use genomic DNA probes, as these may include unwanted intron sequences).

Design of the Oligo Probe

Appendix 1 gives information on the design of primers and probes and how to use GenBank and computer programs to assist in the design process. However, there are a few factors that are omitted from that section, particularly on how to use formamide to adjust the annealing temperature of the probe to a desired temperature.

Many investigators add formamide to their hybridization cocktails to reduce annealing temperature, and each 1% increase in foramide concentration decreases annealing temperature by about 0.62°C (though this is neither an exact function nor a linear one). Most investigators like to have their annealing temperature somewhere in the range of 40°C to 50°C, and a 50% foramide hybridization cocktail is common. Thus for DNA probes used with formamide, the formula commonly used for calculating T_m (midpoint temperature) is the following:

$$T_m = 81.5°C + 16.6\ (\log M) + 0.41\ (\text{percent GC}) - 500/n - 0.62\ (\text{percent formamide})$$

where n is the length of the primers (omit the 500/n factor entirely for any primer or probe less than 100 bp in length) and M is the molarity of the salt in the buffer, usually 0.047 M for DNA reactions.

RNA probes from more stable hybrids than DNA probes. Thus if you are using an RNA probe, the T_m is higher, and the formula for RNA–DNA hybrids is the following:

$$T_m = 79.8°C + 18.5\ (\log M) + 0.58\ (\text{percent GC of probe}) + 0.0012\ (\text{percent GC of target overall}) - 820/n - 0.5\ (\text{percent formamide})$$

where the formamide factor for RNA–RNA hybrids should be 0.35 (percent formamide). Use these formulas to better calculate T_m for hybridization probes, including riboprobes.

Time, Temperature, and Concentration

As can be seen from the calculation above, an investigator has various tools available to adjust the annealing conditions. But changing one parameter affects the others, and there must be an overall optimization to the type of probe being used and the target being detected. Due to the abundance of amplicons, stringent hybridization conditions can be used to achieve high specificity and low background. This means high annealing temperatures (we typically use temperatures only 5° to 10°C below the T_m for the probe in question). Alternatively, the salt concentration can be varied (lower salt means higher stringency at any one temperature) or the content of formamide can be varied in the hybridization cocktail. However, we never try to design hybridization conditions with an annealing temperature below 37°C, nor do we generally go above 55°C.

Finally, the probe concentration and incubation time, two factors that are intimately linked, must be determined. Once again, we favor stringent conditions. If adequate time exists, we use a low probe concentration—20 to 25 pM—with an 18-h or overnight incubation. If there is a rush, we will use a 4-h incubation, but we will raise the concentration only to 40 pM. These low concentrations of probe ensure the highest specificity and lowest background.

Labeling

Both radiolabeled probes and nonradioactively labeled probes may be used, though we prefer the latter. Since there is such an abundance of amplicons present after PCR, there is no need for the higher sensitivity provided by the radioactivity, and nonrad probes work just fine. In fact, we find that end-labeled probes are usually all that are needed. However, if a stronger signal is desired, we will sometimes use probes whose label is conjugated at many sites during synthesis and not merely tail-end conjugated (oligo probes can be ordered with labels attached in this manner, and such probes can also be created through PCR methodologies). The multiple labels supply many more binding

sites per probe during development, which leads to easier detection and stronger signals.

A typical set of labels we will use when multiplexing signals will be a fluorescein–isothiocyanate (FITC) labeled probe to detect a signal amplified from RNA, then a biotinylated probe developed with streptavidin–peroxidase to detect a signal amplified from DNA, then rhodamine-conjugated antibody to detect a protein antigen with immunohistochemical techniques. We counterstain this sample with Gill No. 3 hematoxylin, which imparts a blue color to the negative cells. Other fluorochrome-labeled probes could be added in addition to this (there are at least 20 colors currently available), but the filters to distinguish these fluorochromes are quite expensive. You really need a digital imaging system to analyze the specific wavelengths of light associated with the different fluorochromes, as they are not readily distinguishable with an ordinary microscope. Thus we generally stick to just the FITC probe without other fluorochrome-labeled probes present, as these results are easier to interpret.

Detection Methods

The choice of detection methods must balance sensitivity with resolution, bearing in mind that certain tissues have a peculiar propensity for high background with some detection systems. For example, some tissues have high levels of endogenous peroxidase, and this can increase nonspecific background levels with peroxidase-based systems such as those that use streptavidin or digoxigenin (this is why an overnight quenching in hydrogen peroxide is recommended for tissues that are to be used with peroxidase-based systems). Similarly, other tissues exhibit high endogenous enzyme activity with alkaline phosphatase or even biotin. These characteristics can lead to high background levels as well and may indicate the need for pretreatment to remove or inhibit these endogenous enzymes.

Controls for Hybridization

As with any scientific methodology, the use of controls is exceedingly important with every form of ISH. There are a number of possible failures that can lead to misleading results, and every investigator should bear the following points in mind.

1. Controls should be included to monitor the hybridization reaction for probe specificity and to assess the level of background. This can be achieved with parallel reactions with similarly processed specimens that do not contain the signal being sought. Specifically, misleading results can sometimes result from short complementary regions within a probe hybridizing unexpectedly to unknown sequences, or sometimes nonspecificity results from low stringency annealing conditions, like the annealing temperature being too low. Therefore, adequate controls must be designed and then examined fully to ascertain whether the probe is annealing to unwanted targets or not.
2. Probes may also bind to samples through other nonspecific means, such as through a charge interaction between proteins and probes, particularly with proteins that normally bind DNA. This can be controlled by hybridizing an extra experimental specimen with a similar but noncomplementary probe.
3. With radioactive detection methods, autoradiography artifacts can sometimes yield what look like convincing hybridization signals when in fact no signal is present. An example is chemography, where a chemical reaction between cellular components of a sample and the photographic emulsion produces a pattern of silver grains that look like positive results, even though the radioactive probe is not even present. This can usually be controlled by processing an extra experimental specimen, but one that was hybridized with a nonradioactive version of the probe.

LABELING OLIGONUCLEOTIDE PROBES

There are several methods available for labeling the probes, which are detailed below. We always prefer nonradioactive probes because with in situ PCR, there are so many amplicons to detect that there is no need for the additional sensitivity that radioactivity provides. If you want to use radioactive probes, we strongly recommend ^{33}P over ^{32}P or ^{35}S, because ^{32}P is unnecessarily hot and short-lived, and ^{35}S is associated with labeled chemical breakdown products when used with nucleotides at elevated temperatures. ^{3}H can be quite effective, and it gives the best autoradiographs—but exposures can last 1 month.

Protocols

^{33}P Labeling of Probe For investigators who wish to use radioactivity, here is a typical procedure for the ^{33}P labeling of a probe through a kinase reaction:

2 μM probe	1.0 μL
10× kinase buffer	2.0 μL
ATP g ^{33}P (Amersham 10 μCi/μL)	1.0 μL
dd H_2O	15 μL
Polynucleotide kinase (10 U/μL)	1 μL
Total Volume	*20 μL*

1. Incubate at 37°C for 30 min.
2. Apply sample to 0.8 mL Sephadex G-50 column [eg, QuickSpin (Boehringer, Indianapolis, Ind.)].
3. Elute with TE buffer.

Fraction 1	300 μL
Fraction 2	100 μL
Fraction 3	100 μL
Fraction 4	100 μL
Fraction 5	100 μL
Fraction 6	100 μL

4. Count the radioactivity in 1.0 μL of each fraction. The labeled probe should be contained in fractions 2 through 4.

Nonradioactive Labeling-tailing with DIG-11-dUTP Many laboratories, including our own, always use nonradioactive probes, for this results in less hazardous waste, fewer bureaucratic procedures, and lower costs (especially considering the perishable nature of radioactive isotopes and probes). The following is a typical labeling procedure.

20 μM probe	10 μL
5 × tailing buffer (see note)	10 μL
25 mM $CaCl_2$	20 μL
2.5 mM dATP (tris buffer pH 7.5)	3.5 μL

1 mM DIG-11-dUTP	1.0 μL
dd H_2O	4.5 μL
Terminal transferase (25 U/μL)	1.0 μL
Total Volume	*50 μL*

Note: Tailing buffer (5×): 1 mM potassium cacodylate, 125 mM tris-HCl, pH 6.6, and 1.25 mg/mL BSA.

1. Incubate reaction mixture at 37°C for 15 min; then purify the labeled probe as for the ^{33}P probe, using a chromogen indicator instead of radioactive detectors.

We prefer to use biotinylated probes, which we purchase already conjugated. These probes are either tail-end conjugated or the labels are incorporated during the oligo-synthesis procedure. The latter type contains multiple molecules of biotin instead of one at the tail end, which makes the probes more sensitive. Many other nonradioactive nucleotides are commercially available and can be labeled in a similar manner. The probes are good for up to 1 year at −70°C.

PROTOCOLS: HYBRIDIZATION METHODS FOR IN SITU PCR

General Hybridization

Prepare a solution containing: 20 pM of the appropriate probe(s), 50% deionized formamide, 2× SSC buffer, 10× Denhardt's solution, 0.1% denatured sonicated salmon sperm DNA, and 0.1% SDS. The following is a convenient recipe.

Probe(s) (^{33}P, FITC, fluorochromed, biotinylated digoxigenin, or combinations thereof)	20 pM each (adjust dd H_2O)
Deionized formamide	50 μL
20× SSC (see note)	10 μL
50× Denhardt's solution	20 μL
10 mg/mL ssDNA (see note)	10 μL

10% SDS	1 μL
dd H_2O	9 μL (minus the volume of probe solution)
Total Volume	*100 μL*

Note: See Chapter 8 for preparation of 20 × SSC buffer

Note: The salmon sperm should be denatured at 94°C for 10 min before it is added to the hybridization buffer

Note: 2% BSA can be added as an additional blocking agent if you observe nonspecific binding. For this purpose, add 10 μL of 20% BSA solution and reduce the amount of water accordingly.

1. Add 10 μL of hybridization mixture to each well of a three-chamber slide or 30 μL to a whole slide. Lay cover glasses over the reaction.

2. Heat the slides on a heat block at 95°C for 5 min. Alternatively, program the thermal cycler for the denaturation followed by the hybridization (if the cycler has humidification).

3. Incubate the slides at 37° to 42°C for a minimum of 18 h in a humidified atmosphere. Of course, the incubation temperature used should be adjusted to the optimal temperature for the probe(s) being used (this temperature has been optimized for the SK-19 probe, which detects HIV-1). If you are in a hurry, the concentration of the probe(s) can be doubled and the incubation time reduced to 4 h.

It is necessary to use a humidified incubator for this long incubation, or the slides must be sealed in some manner to keep them from drying out. The only thermal cycler that we have found to be useful for this purpose is the PTC-200 Twin Tower, which holds 32 slides. If you put a wet Kimwipe or sponge into the bottom slot, sufficient water vapor is generated to keep the slides from drying out during an overnight incubation.

The optimal hybridization temperature is a function of the T_m of the probe, and we usually use a temperature of $T_m - 5$°C. However, the hybridization temperatures used should never be too high (>75°C); we rarely go above 55°C. If using multiple probes, try to design them so they all have a similar T_m, then use the lowest hybridization temperature among the probes in the hybridization cocktail.

Posthybridization for ^{33}P Probe

1. Wash the slides in 2× SSC for 5 min.

Note: Steps 2 to 5 should be carried out in the dark.

2. Dip the slides in 3× nuclear tract emulsion (Kodak NBT-2, diluted 1:1 with water).
3. The slides are air dried, and then incubated for 3 to 10 days in a light-proof box with a drying agent (period of incubation depends on the concentration and counts per minute of the probe).
4. The slides are developed for 3 min in Kodak D-19 developer, and then rinsed in dd H_2O.
5. The slides fixed for 3 min in Kodak Unifax.
6. Counterstain the slides with May Grunewald stain (blue) or nuclear fast red. Mount the slides with Clear Mount reagent.

Posthybridization for FITC or Other Fluorochrome-labeled Probes

1. Wash the slides twice in 1× PBS.
2. No other posthybridization procedure is needed to develop fluorescein–isothiocyanate (FITC) or other fluorochrome-labeled probes, as they already possess their characteristic colors. Currently, there are more than 20 fluorochromes available, which can be conjugated with different probes to show different signals. However, special filters are required to distinguish each color, and these filters are quite expensive.
3. Examine the slides under a microscope to ascertain that hybridization has been successful. Be sure to examine the negative control particularly to make sure there has not been any nonspecific hybridization.
4. If another probe in the experiment requires development, perform posthybridization at this point. If you are performing immunohistochemical detections, react the tissue with appropriate rhodamine-conjugated antibody.
5. Wash the slides gently in 1 × PBS and counterstain with Gill No. 3 hematoxylin. This will impart a blue color to the negative cells without adding any fluorescence.

6. Place the cover glasses and mount the slides. We prefer to mount the slides with a solution of 50% glycerol in PBS and store them under refrigeration at 4°C; this solution preserves the signals without leaching. For longer storage, the cover glass can be adhered around the periphery with nail polish or rubber cement, but under no circumstance should you store the slides at freezing temperatures.

Posthybridization for Peroxidase-based Color Development

Many different types of probes can be used so that peroxidase-based colors can be detected. These include biotinylated probes that can be bound with streptavidin–peroxidase and digoxigenin probes that can be reacted with antidig antibody conjugated with peroxidase. The development procedure provided here is for the biotin–streptavidin system.

1. Wash slides in 2× SSC for 5 min. Wash slides in 1× PBS for 5 min.
2. Add 10 μL of streptavidin–peroxidase complex (π/mL PBS pH 7.2) diluted 1:30 in 1× PBS. Gently apply the cover glasses.
3. Incubate the slides at 37°C for 1 h in a humidified incubator.
4. Remove the cover glass, wash the slides with 1× PBS twice for 5 min each time.
5. Add to each well 100 μL of 3′-amino-9-ethylene carbazole (AEC) in the presence of 0.03% hydrogen peroxide in 50 mM acetate buffer (pH 5.0). No cover glasses are required, but be sure there is adequate fluid to cover the sample.
6. Incubate the slides at 37°C for 10 min to develop the color; *this step should be carried out in the dark*. After this period, observe slides under a microscope. If the color is not strong, develop for another 10 min.
7. Rinse the slides with tap water and allow them to dry.
8. Add 1 drop of 50% glycerol in PBS, and apply the cover glasses (do not counterstain yet).
9. Examine with an optical microscope: positive cells will be stained a brownish red and negative cells will be colorless. Be sure to carefully examine the control slides, particularly the negative control to confirm it is completely colorless. Sometimes the negative control will show staining due to the presence of endogenous peroxidase (insufficient hydrogen

peroxide treatment before amplication) or endogenous biotin (particularly in liver or tumor cells). If there is any staining of the negative control due to endogenous peroxidase or biotin or there are "rim stains" around the cells in the mixed population due to leakage of signal from the positive cells, the reaction has failed and the experiment must be repeated.

10. If you are using immunohistochemical techniques, react the tissues with the appropriate antibody at this point. Usually, we will use rhodamine-conjugated antibodies for immunohistochemistry, as we save FITC and fluorochrome labeling for other oligo probes we may be using.

11. Wash the slides gently in 1× PBS and counterstain with Gill no. 3 hematoxylin, which will impart a blue color to negative cells without adding any florescence.

12. Place the cover glasses and mount the slides. We prefer to mount the slides with a solution of 50% glycerol in PBS and store them under refrigeration at 4°C, as this solution preserves the signals well without leaching. For longer storage, the cover glass can be adhered around the periphery with nail polish or rubber cement. Under any circumstances, do not freeze the slides.

Posthybridization for Alkaline–Phosphatase Color Development

1. After hybridization, remove the cover glasses and wash the slides with two soakings in 2× SSC at room temperature for 15 min.

2. Cover each well with 100 µL of blocking–bleaching solution (see below) and place the slides flat in a humidified chamber at room temperature for 15 min.

3. Prepare a working conjugate solution by mixing 10 µL of streptavidin–alkaline phosphatase conjugate (40 µg/mL stock) with 90 µL of conjugate dilution buffer (see below) for each well.

4. Remove the blocking solution from each slide by touching a paper towel to the edge of the slide.

5. Cover each well with 100 µL of freshly prepared working conjugate solution and incubate in the humidified chamber at room temperature for 15 min. Do not allow the tissue to dry out after adding the conjugate, though cover glasses are not required.

6. Wash slides by soaking in buffer A (see below) for 15 min at room temperature, two times.

7. Wash slides once in alkaline substrate buffer (see below) at room temperature for 5 min.

8. Prewarm 50 mL of alkaline-substrate buffer (see below) to 37°C in a Coplin jar. Just before adding the slides, add 200 μL NBT and 166 μL of BCIP (see below). Mix well.

9. Incubate the slides in the NBT–BCIP solution at 37°C until the desired level of signal is achieved (usually 10 min to 2h). Check the color development periodically by removing a slide from the NBT–BCIP solution. Be careful not to allow the tissue to dry out.

10. Stop the color development by rinsing the slides in several changes of deionized water.

11. Examine the slides under a microscope, particularly the controls. If the negative control shows any nonspecific staining, usually blue rims around the cells, the reason might possibly be endogenous alkaline–phosphatase in the tissue that was not bleached out in Step 2. The experiment must be repeated, but next time try doubling the incubation in Step 2 to 30 min (the levamisole will bleach the endogenous alkaline phosphatase in mammalian cells but not the prokaryote-derived alkaline phosphatase in the probe).

12. If you are using immunohistochemical techniques, react the tissues with the appropriate antibody at this point. Usually, we will use rhodamine-conjugated antibodies for immunohistochemistry, as we save FITC and fluorochrome labeling for other oligo probes we may be using.

13. Wash the slides in 1× PBS and counterstain with nuclear fast red.

14. Place the cover glasses and mount the slides. We prefer to mount the slides with a solution of 50% glycerol in PBS and store under refrigeration at 4°C, as this solution preserves the signals well without leaching. For longer storage, the cover glasses can be adhered around the periphery with nail polish or rubber cement, but do not freeze the slides.

Blocking–bleaching solution: 50 mg/mL BSA (protein) and 1% levamisole (pH 8.1 in 1× PBS) in 100 mM tris-HCl (pH 7.8), 150 mM NaCl, and 0.2 mg/mL sodium azide

Conjugate dilution buffer: 100 mM tris-HCl, 150 mM $MgCl_2$, 10 mg/mL BSA, and 0.2 mg/mL sodium azide

Buffer A: 100 mM tris-HCl (pH 7.5) and 150 mM NaCl

Alkaline substrate buffer: 100 mM tris-HCl (pH 9.5), 150 mM NaCl, and 50 mM $MgCl_2$

NBT (Nitro-blue-tetrazolium): 75 mg/mL NBT in 707% (v/v) dimethylformamide, freshly prepared

BCIP (4-bromo-5-chloro-3-indolylphosphate): 50 mg/mL in 100% dimethylformamide, freshly prepared

Posthybridization for Digoxigenin-labeled Probe

1. Use an antidig–peroxidase solution (Boehringer-Mannheim), 1 mg/mL, 1:250 dilution in PBS. Incubate the slides for 2 h at 37°C in a humidified incubator. Wash the slides three times with PBS; then develop the color with AEC as with other peroxidase-based probes, beginning at Step 5, page 000.

POSTHYBRIDIZATION OF DIRECTLY INCORPORATED LABELED NUCLEOTIDES

Color development and solution formulations can be selected from the above recipes. For example, if you used biotin-labeled nucleotides, then streptavidin–peroxidase or alkaline phosphates can be used as secondary labels. If digoxigenin was used, then antidig with peroxidase, alkaline phosphatase, or FITC can be used to observe the signals.

CHAPTER 7

VALIDATION AND CONTROLS

The validity of in situ amplification–hybridization should be examined in every run. Attention here is particularly important in laboratories using the in situ technique for the first time, because many technical pitfalls lie on the path to mastery. In an experienced laboratory such as our own, it is still necessary to continuously validate the procedure and to confirm the efficiency of amplification. To do this, we routinely run two to three sets of experiments simultaneously (each with a positive and negative control), for we must not only validate amplification but also confirm the subsequent hybridization and detection steps as well.

We frequently work with HIV-1. A common validation procedure we conduct is to mix HIV-1-infected cells plus HIV-1-uninfected cells in a known proportion (eg, 1:10 or 1:100), then we confirm that the results are appropriately proportionate. This technique also provides an opportunity to look for leakage of the amplicons and their penetration into the surfaces of adjacent, HIV-negative cells. After the in situ PCR and hybridization procedures, we carefully scan the entire slide under a high power microscope and search for periplasmic colors (which we also call rim staining). These are the unwanted signs that indicate signal leakage has occurred.

To examine the efficiency of amplification, we use cell lines that are known to carry one or two copies of cloned HIV-1. Following PCR and

hybridization, we look to see that proper amplification and hybridization has occurred in both the experimental samples and the controls. You do not have to use HIV-1 cell lines; any endogenous genes could be used to detect the efficiency of in situ PCR, including HLA-DQa, β-actin or β-globulin genes. HLA-DQ genes exist in a single copy per cell in most of the human cells, since there is usually only a single haplotype per cell. The two latter genes generally exist as two copies per cell (ie, each pair of chromosomes carries one copy apiece).

In all DNA PCR procedures, we include at least two slides to be used as controls for nonspecific binding and development of the hybridization probe. We hybridize one slide of amplified cells with an unrelated probe. The second slide would have been put through the PCR procedure without *taq* polymerase, and we hybridize that slide with the proper probe. The former slide should show no staining at all, and the latter slide should show little or no visible staining (unless the cells contain a high copy number of the gene per cell). Sometimes we also process slides in parallel with primers and probes for HLA-DQa and β-actin, especially with human PBMCs. These serve as positive controls, and we use them to check various parameters of our system.

If you are using tissue sections with in situ PCR, a cell suspension lacking the gene of interest can be used as a control. These cells can be added on top of the tissue section and then can be retrieved after the amplification procedure or left as they are for further processing. These cells usually come to rest within crevices of the tissue sections, and they will reveal if any leakage has taken place. With careful handling, the cell suspension can be hybridized and developed with the tissue section, or the cells can be recovered with the supernatant by the various means described on pages 25–28. Then the cells must be hybridized and developed separately. If the signal from the tissue has leaked out in any appreciable quantity, it will generally be found in these cells (particularly as the rim staining phenomenon).

One question often asked is where does one get cell lines lacking the genes of interest? There are several potential sources. For example, if one is working with endogenous genes of a mammal, then nonmammalian cells would probably be a good source, particularly if the gene of interest has not been highly conserved through evolution. Otherwise, phylogenetically lower species can be used, like invertebrate species, or marine species. The ATCC and several other cell and tissue banks store and col-

lect many cultures of biologic material, and samples from these collections can be obtained quite readily. The other increasingly rich source are knockout mice, which are devoid of certain gene(s). This is an emerging technology, and one that should serve as an increasingly abundant source in the future.

In the case of RT in situ PCR, you can use β-actin, HLA-DQa, and other endogenous-abundant RNAs as the positive markers. Of course, you should always have a negative control for RT in situ amplification, as well as controls for the DNAase reaction if a DNAase procedure was used. Controls without *taq* polymerase plus primers and with *taq* polymerase and without primers should be included as well. In case of r*Tth* protocols, cocktail without $MnCl_2$ should be used as one of the negative controls.

Investigators should always put considerable thought and effort into the design of proper positive and negative controls for their specific experiment. Then they must use the controls, diligently analyze the results, and realize that every investigator periodically experiences failed reactions. It is all part of science.

CHAPTER 8

MATERIALS AND METHODS

SLIDES

Glass slides suitable for this procedure are available from several sources. Please remember that if using the nail polish sealing technique, a heavy Teflon coating that forms individual wells is best for this protocol. Either purchase slides with untreated glass and silanate the slides yourself with the AES solution specified within 60 days of use, or purchase slides with the manufacturer's special coating.

There are three basic designs from which to choose for this purpose: slides with a single oval well, which are better for tissue sections; slides with three 12- or 14-mm wells, which are better for cell suspensions and cell smears; and slides with twelve 6-mm wells, which can be used with particularly scarce tissue, like fetal tissue. However, please bear in mind that unwanted artifacts sometimes appear with small wells, so use the larger wells whenever possible. Here is the ordering information for three sources.

Manufacturer	Cell-Line Associates, Inc. P.O. Box 648 Newfield, N.J. 08344 800-662-0973/ fax: 609-697-9728	Erie Scientific 20 Post Road Portsmouth, N.H. 03801 800-258-0834/ fax: 603-431-8996	MJ Research, Inc. 149 Grove Street Watertown, Mass. 02172 800-729-2165/ fax: 617-923-8080; World Wide Web: **www.mjr.com**

Teflon-coated slides			
Single-well	#10-503	#ER-203	—
Three-well	#10-12	#ER-285	—
Twelve-well	#10-103	#ER-297	—
Slides for use with Self-Seal reagent			
Without wells	—	NA	Address as above
Single-wells	—	NA	Address as above
Three-well	—	NA	Address as above

COPLIN JARS AND GLASS STAINING DISHES

Suitable vessels for washing, fixing, and staining 4 to 50 glass slides are available from several vendors. It is best to obtain dishes with a similar capacity to your thermal cycling apparatus. Dishes with removable trays that hold the slides apart are particularly convenient. Such vessels are available from Fisher Scientific, VWR Scientific, and Baxter, and they are generally listed as staining dishes or Coplin jars.

SOLUTIONS AND REAGENTS

2% Paraformaldehyde

1. Take 12 g paraformaldehyde (Merck ultra pure Art no. 4005) and add to 600 mL 1× PBS.
2. Heat at 65°C for 10 min.
3. When the solution starts to clear, add 4 drops 10 N NaOH and stir.
4. Adjust to neutral pH (7.2 to 7.4) and cool to room temperature.
5. Filter on Whatman's no. 1.

10× PBS Stock Solution pH 7.2 to 7.4

Dissolve 20.5 g $NaH_2PO_4H_2O$ and 179.9 g $Na_2HPO_47H_2O$ (or 95.5 g Na_2HPO_4) in about 4 L double-distilled water. Adjust to the required pH (7.2 to 7.4). Add 701.3 g NaCl and make up to a total volume of 8 L.

1× PBS

Dilute the stock 10× PBS at 1:10 ratio (ie, 100 mL 10× PBS and 900 mL of water for 1 L). The final concentration of the buffer should be 0.01 M phosphate and 0.15 M NaCl.

0.3% Hydrogen Peroxide (H_2O_2) in PBS

Dilute stock 30% hydrogen peroxide (H_2O_2) at a 1:100 ratio in 1× PBS for a final concentration of 0.3% H_2O_2.

Proteinase K

Dissolve powder from Sigma in water to obtain a concentration of 1 mg/mL. Aliquot and store at −20°C. Working solution: dilute 1 mL of stock (1 mg/mL) into 150 mL of 1× PBS.

20× SSC

Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 mL water. Adjust the pH to 7.0 with a few drops of 10 N solution of NaOH. Adjust the volume to 1 L with water. Sterilize by autoclaving.

2× SSC

Dilute 20× SSC; 100 mL of 20× SSC and 900 mL of water.

Solutions for Amplifying Chromosomal Bands

1. RPMI medium 1640: Per 100 mL, supplement with 15 mL fetal bovine serum, 1.5 Hepes buffer (IM), 0.1 mL Gentamicin (0.1 heparin is optional)
2. Velban: Reconstitute vial with 10 mL sterile H_2O. From this solution, dilute 0.1 mL into 50 mL distilled H_2O. Store in the refrigerator.
3. EGTA hypotonic solution: Dissolve 0.2 g EGTA powder, 3.0 g KCL, and 4.8 g Hepes buffer into 1000 mL of distilled H_2O. Adjust the pH to 7.4. Store in the refrigerator and, before use, prewarm to 37°C.
4. PHA-C (phytohemagglutinin): Reconstitute with 5 mL sterile H_2O. Aliquot into five 1-mL insulin syringes. Freeze four for later use, leave one in the refrigerator.
5. Fixative solution: Mix 1 part glacial acetic acid in 3 parts EM-grade methanol, store at −20°C.

Streptavidin Peroxidase

Dissolve powder from Sigma in PBS to make a stock of 1 mg/mL. Just before use, dilute the stock solution in sterile PBS at a 1:30 ratio.

Color Solution

Dissolve one AEC (3-amino-9-ethyl-carbazole from Sigma) tablet in 2.5 mL of *N,N*,dimethyl formamide. Store at 4°C in the dark.

Working Solution	
50 mM acetate buffer (pH 5.0)	5 mL
AEC solution	250 µL
30% H_2O_2	25 µL

Make fresh before each use; keep the solution in the dark.

Preparation of 50 mM Acetate Buffer, pH 5.0

Add 74 mL of 0.2 N acetic acid (11.55 mL glacial acid/L) and 176 mL of 0.2 M sodium acetate (27.2 g sodium acetate trihydrate in 1 L) to 1 L of deionized water and mix.

In situ Hybridization Buffer (for 5 mL)

Formamide	2.5 mL
Salmon sperm DNA (ssDNA) (10 mg/mL; see note)	500 µL
20× SSC	500 µL
50× Denhardt's solution	1 mL
10% SDS	50 µL
H_2O	450 µL (minus volume of probe solution)
Total volume	*5 mL*

Note: Heat denatured ssDNA at 94°C for 10 min before adding it to the solution. Save aliquots in 1-mL volumes and freeze at −70°C. Add probe(s) at the time of hybridization. Properly stored, this solution should remain good for up to 3 years.

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APPENDIX I

COMPUTER-ASSISTED DESIGNING OF PRIMERS AND PROBES

This appendix deals with the use of computers in designing the primers and probes needed for the procedures outlined in this volume. Before selecting a primer pair for in situ PCR, it is helpful to become familiar with various computer software programs that will simplify some of the research tasks. The first step in primer or probe design involves obtaining the sequences of the gene of interest from the appropriate database. The sequences are then input into the software program, which assists in choosing the optimal sequences for in situ PCR or hybridization.

SEQUENCE RETRIEVAL FROM GENBANK

Our knowledge regarding DNA sequences has grown enormously with the advent of modern sequencing facilities and the initiation of the Human Genome Project. At the same time, computer programs for analyzing these data have become more sophisticated. There are a number of databases that are easily accessible to molecular biologists via the Internet and the World Wide Web (WWW). The Internet has also become an important tool for retrieving software, obtaining up-to-date scientific information, and connecting with other research organizations.

Genbank is the most comprehensive database of DNA and protein sequence information. It is sponsored by the National Institutes of Health (NIH) and is distributed by the National Center for Biotechnology Information (NCBI). GenBank works in collaboration with the European Bioinformatics Institute (EBI) and the DNA Data Bank of Japan (DDBJ).

There are a number of ways to access GenBank's databases via the Internet and WWW. Two common Internet and WWW browser programs are *Netscape* and *Mosaic*. Each DNA and protein sequence submitted to GenBank is assigned a unique accession number and given a few characterizing qualifiers, which are used to retrieve it.

The following example shows how to retrieve the simian immunodeficiency virus (SIV) *gag* gene, which can be used to design a primer for either analytical or in situ PCR. The Macintosh program used in the exam-

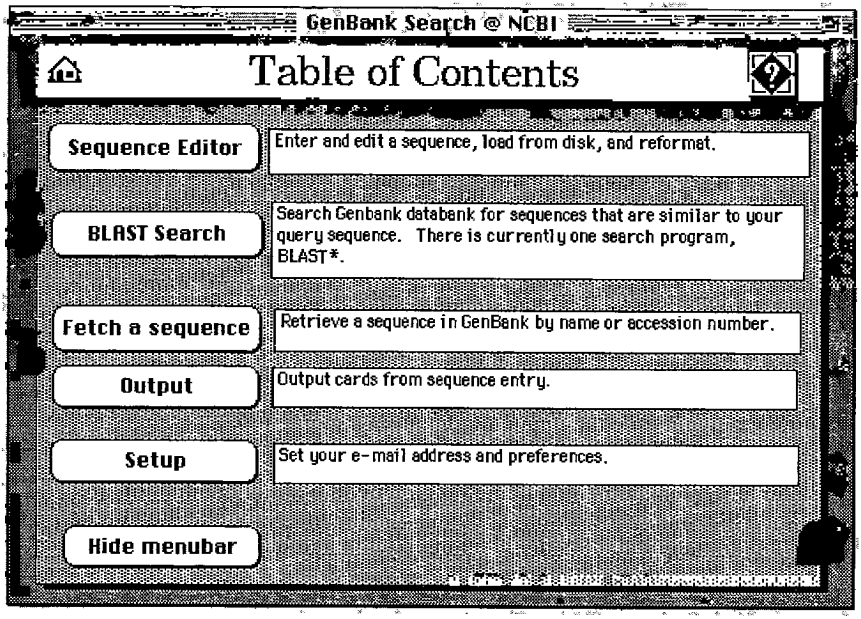

FIGURE A1.1. The "Table of Contents" screen pops up after you initiate the GenBank *Fetch* program. The researcher has the option to retrieve a sequence or to search a sequence's homology with other sequences in the GenBank database. It is important to enter the "Setup" menu to provide an e-mail address to which the requested information is sent.

ple can be retrieved through e-mail from Software@bio.indiana.edu. This program has the ability to search (via *BLAST*) and retrieve data from GenBank (the data can also be retrieved via *Netscape* at http://www.ncbi.nlm.nih.gov/). To use the GenBank *Fetch* software, you must have an e-mail address and, of course, be connected to the Internet.

The initial screen after opening *Fetch* is the "Table of Contents" (Fig. A1.1), which provides the researcher with a number of options, including searching via *BLAST* and retrieving a sequence via *Fetch*. In the "Setup" menu, provide a return e-mail address. To retrieve a sequence, click on "Fetch a sequence."

If you know the accession number—for the SIV *gag* gene, the accession number is U17646—type it in the "Locus name" dialogue box, and then click on "Fetch It!" (Fig. A1.2). Within a few minutes, the information is mailed back to the e-mail address provided in the "Setup" menu described earlier. If you do not know the accession number, you can type in the author's name or the name of the organism to get the same information.

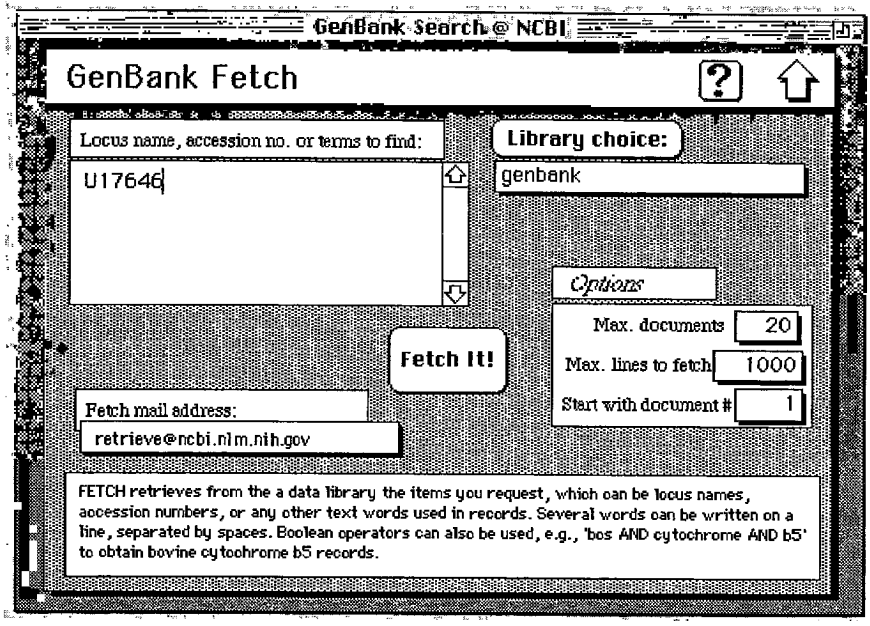

FIGURE A1.2. The "Search" screen allows the researcher to retrieve a sequence by typing in the accession number, the author's name, the locus name, or the name of the organism.

PRIMER DESIGN:

Selection of a particular target gene is followed by choosing a primer pair that can be used to amplify a segment of genetic material. There are a number of programs available for both the Macintosh and PCs. Our laboratory uses the Macintosh version of *Right Primer*. Before choosing primers for in situ PCR, certain important points must be kept in mind. In situ PCR follows the same principles as conventional PCR, except that the DNA or RNA strand to be amplified is inside fixed cells. Each cell simulates a microvessel tube used in conventional solution PCR. Furthermore, permeability and retention of primers, dNTPs and polymerase are also important. It has been our experience that for in situ PCR shorter template amplification provides better results as described in detail earlier.

The following example describes how a primer can be designed using *Right Primer*. The initial step is the selection of the gene that will be used as an identification marker. The SIV *gag* gene (Chen et al. 1995) will again be used to demonstrate primer designing and selection.

Right Primer is initiated by clicking on *Right Primer* 1.1 (shown in the figure on top left corner) (Fig. A1.3). This will display a window that

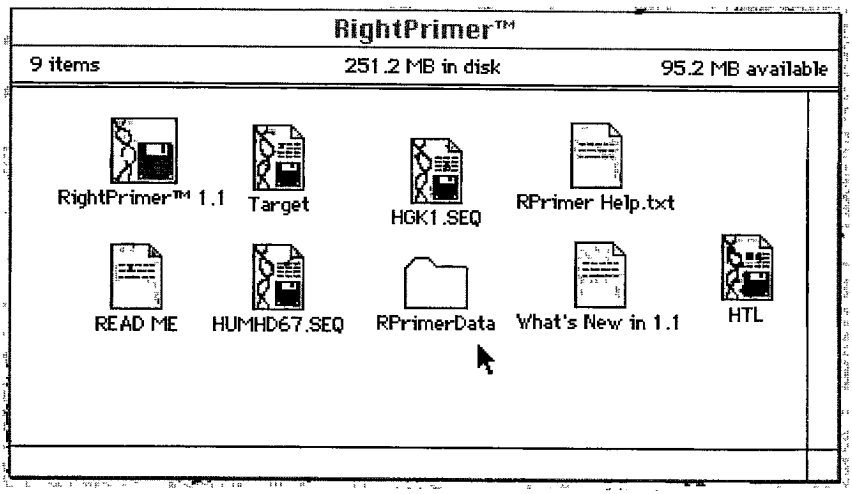

FIGURE A1.3. *Right Primer* window showing the icon for starting the program as well as icons for the program's accessories.

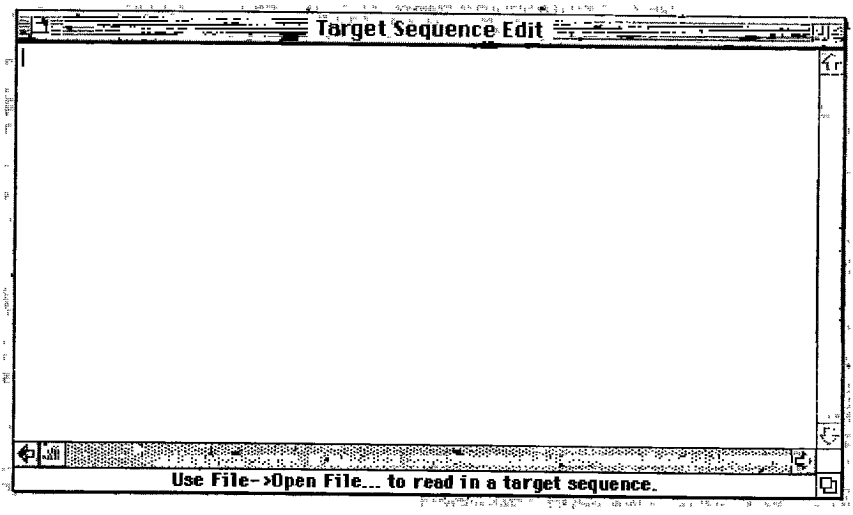

FIGURE A1.4. Open box where target sequences for primer selection are copied to continue with the remaining steps of the *Right Primer* program.

looks like the following (Fig. A1.4). The gene sequences are copied into this window using the "Edit" menu. After copying the sequences in the blank window, retrieved via Fetch or through Netscape software, the following steps are performed to select the primers.

1. For formatting the bases, position the cursor before the first base and click the mouse twice. This will format the bases for analyses and display them in groups of 10, with the base number on left side of the window (Figs. A1.5 and A1.6).

2. Next, click on "Select Primers" in the PCR parameters menu. A dialogue box prompts you to select a number of parameters, such as the number of sense and antisense primers, melting temperature for each oligo, size in bp, percentage of G and C bases, and maximum allowable base run (ie, consecutive repetition of a given bases). There are a number of options that have been chosen for PCR primer selection of the SIV *gag* gene (Fig. A1.7). For example, the number of primers is set at 10 and the melting temperature at 55°C.

3. Next, from the "Select Primers" menu select GenBank background, which contains oligomer frequencies computed from the GenBank entries for each species or group (Fig. A1.8).

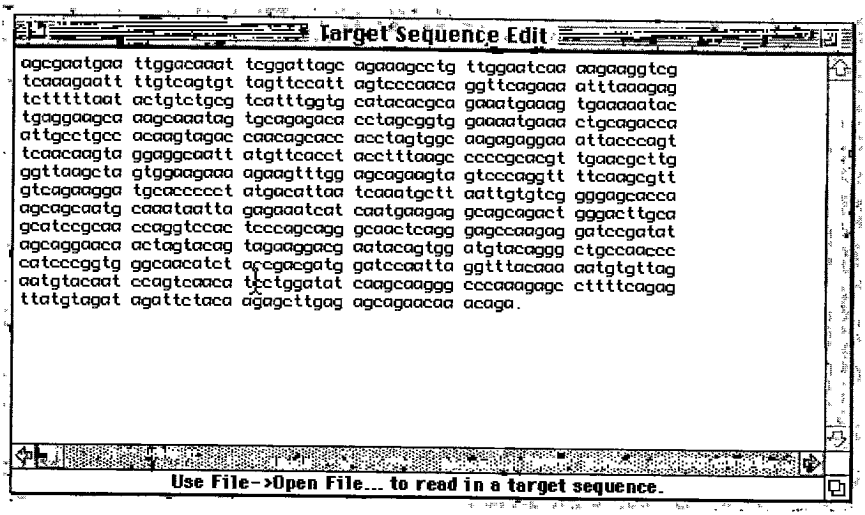

FIGURE A1.5. Target sequences in unformatted form in the *Right Primer* program.

Selection of the GenBank background is followed by selection of a particular primer pair (eg, sense and antisense) that can be used for amplification of a specific length of gene segment. In this example, two primers were selected that amplify a 235 bp fragment (Fig. A1.9).

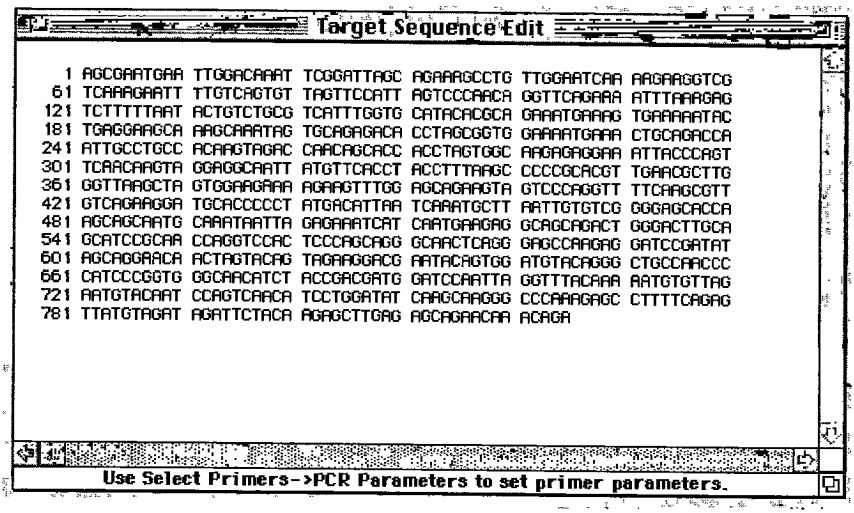

FIGURE A1.6. Formatted sequences for primer analyses.

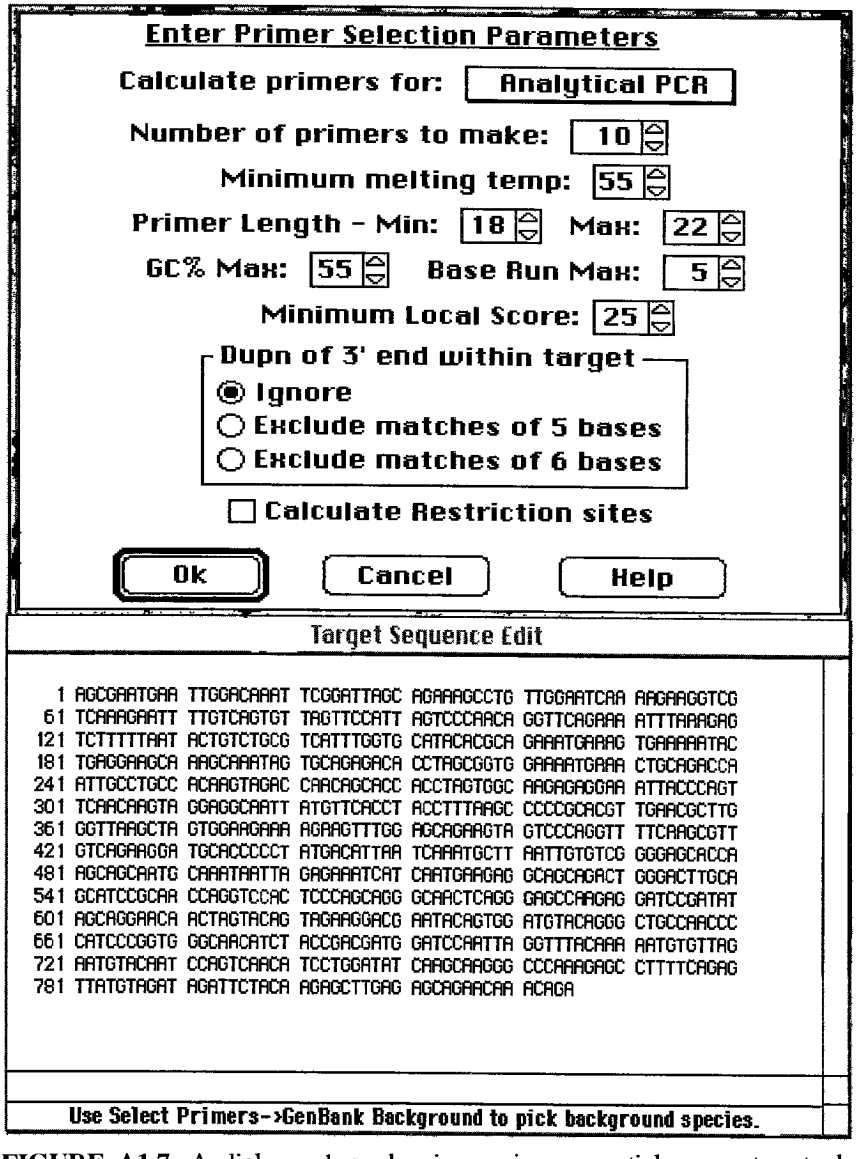

FIGURE A1.7. A dialogue box showing various essential parameters to be kept under consideration for selection of PCR primers.

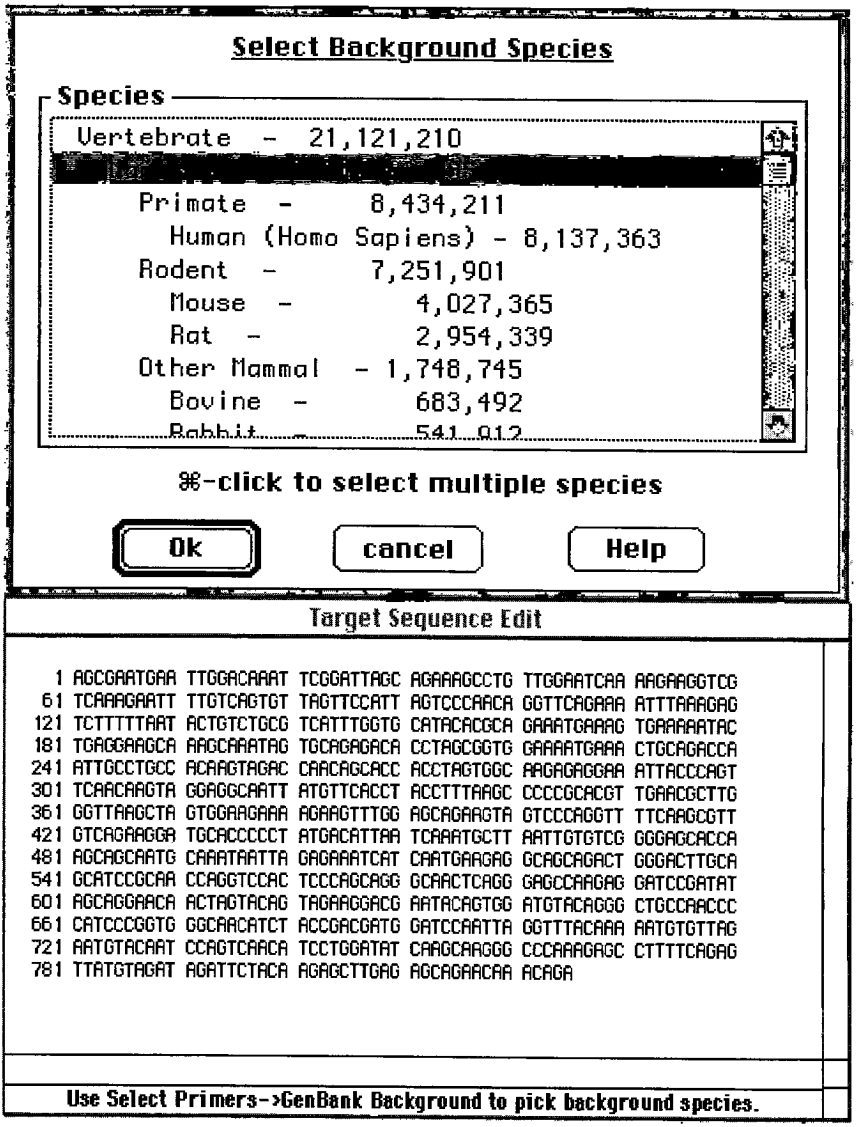

FIGURE A1.8. Selection of GenBank background for the sequences of interest.

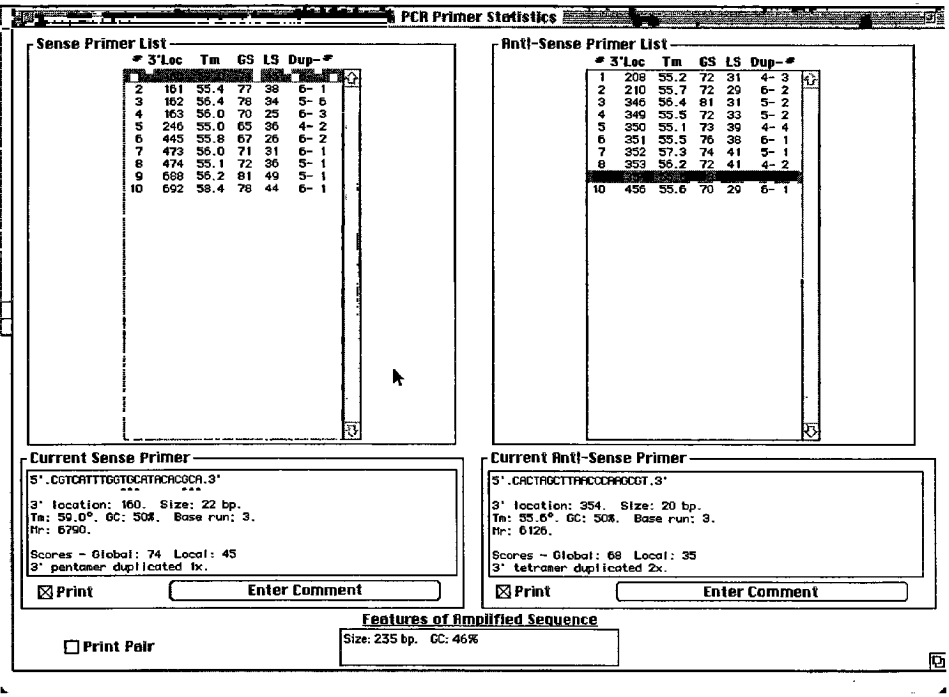

FIGURE A1.9. Figure explaining the selection of a particular sense and antisense primer.

PRIMER OPTIMIZATION

Before starting in situ PCR, primer optimization is essential. It has been observed that primer annealing for a template is optimal at 2°C above its T_m. We hypothesize that primers do not have easy access to DNA templates inside cells and tissues, due to numerous membranes, folds, and other cellular structures that can obstruct their binding to the target. To overcome this problem, for each new set of primers a solution-based reaction is conducted to confirm the best annealing temperature. A thermocycler designed for the determination of actual annealing temperature (Robocycler; Stratagene; La Jolla, Calif.) has provided encouraging results in our laboratory. Besides this, a thermocycler designed by MJ Research that has the capability to perform both in situ gene amplification in slides and in solution simultaneously in the same block is also one of

the best choices for determining the optimal amplification and for running proper controls.

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Contributed by
Muhammad Mukhtar and Omar Bagasra
The Dorrance H. Hamilton Laboratories,
Center for Human Virology
Division of Infectious Diseases
Department of Medicine
Jefferson Medical College
Thomas Jefferson University
Philadelphia, Pa. 19107

APPENDIX 2

AMPLIFICATION: THE DETECTION OF RARE EVENTS

DETECTION OF RARE EVENTS AND ONCOLOGY

Advances in methodology, instrumentation, and computer technology allow us to obtain new answers to old questions and gives us the ability to ask questions that older technologies had not been capable of answering. In situ amplification is one such new methodology. It is of interest to apply immunocytochemistry (ICC), in situ hybridization (ISH), and in situ reverse transcriptase polymerase chain reaction (RT–PCR) to an area that has in the past presented insurmountable technical difficulties. That area is the detection of rare cellular events. This has immediate applications in oncology and infectious disease. We, and others, have designed and constructed high speed, scanning, bright field microscopes capable of automatically detecting a single cellular event in population of $1:1\times10^{6}$ to $1:1\times10^{8}$ cells. Combining the instrument with the appropriate reagents and chromogenic substrates has now opened the door to new areas of medical and scientific investigation.

The advantage of the instrument is to automate the most labor-intensive, tedious part of searching for rare events. That is the actual scanning of the slides to allow the reviewer to make the final decisions as to whether the events detected are true events or false positives. This is accomplished as follows. From 1 to 100 previously stained slides (ICC,

ISH, and/or RT–PCR) are loaded into the microscope input hopper. Using a computer menu, the user inputs the area to be scanned and the name of the chromogen (eg, DAB, AEC, New Fuchsin) and whether the instrument should count all of the nucleated cells (which are hematoxylin stained) to provide a denominator. The automated scan menu button is then pressed, and the scanning of all of the loaded slides begins (Fig. A2.1). At the completion of the run, the reviewer is presented with a visual montage of all of the events detected by the instrument (Fig. A2.2). Since the instrument is set to detect all rare events, it necessarily detects some false-positive events. The reviewer is then able to examine the montage and accept or reject each event image. The instrument then calculates the number of accepted events per 100,000 nucleated cells and generates a report.

We have performed initial experiments to validate this concept. With appropriately stained slides in a spiking experiment the system was able to detect 1 cell per 100 million negative cells (1). Using clinical samples of bone marrow (BM) from a patient with breast cancer, the system was able to detect 1 cell in 14 million nucleated BM cells (2). The use of these methodologies, instrumentation, and computer technology in the detection of minimal residual disease in oncology should result in benefits to the patient and the oncology community. Early primary cancer detection programs (mammography for breast cancer, Pap smears for cervical cancer, and PSA assays for prostate cancer) are a fundamental part of the war on cancer. They are all predicated on the hypothesis that detection of cancer at an early stage, when there is minimal disease and when the genetic instability of tumors is at a minimum, can lead to curative therapy, whereas at later stages, when there is a large tumor mass and many different clones have arisen, cures are rare. For example, 90% of Stage I ovarian cancer is surgically curable, but at later stages the cure rate is only 15%. We believe that a similar situation holds in other surgically resectable tumors such as lymph node negative breast cancer, Duke's Class B and C colorectal cancer, Stages I to III lung cancer, and organ-confined prostate cancer. In all of these situations, surgery leads to a presumptive cure. However, in each of these cases there is a 30% chance of tumor recurrence. The medical literature has amply demonstrated that detection of the presence of tumor cells ($1:1\times10^5$ to $1:1\times10^6$ BM cells) in the BM is an independent prognostic indicator for shortened disease-free survival as well as overall survival (3, 4). The rationale of detection of early primary cancer to enable curative therapy

FIGURE A2.1. Preference screen. The automated scanning menu button is then pressed and the scanning of all of the loaded slides begins.

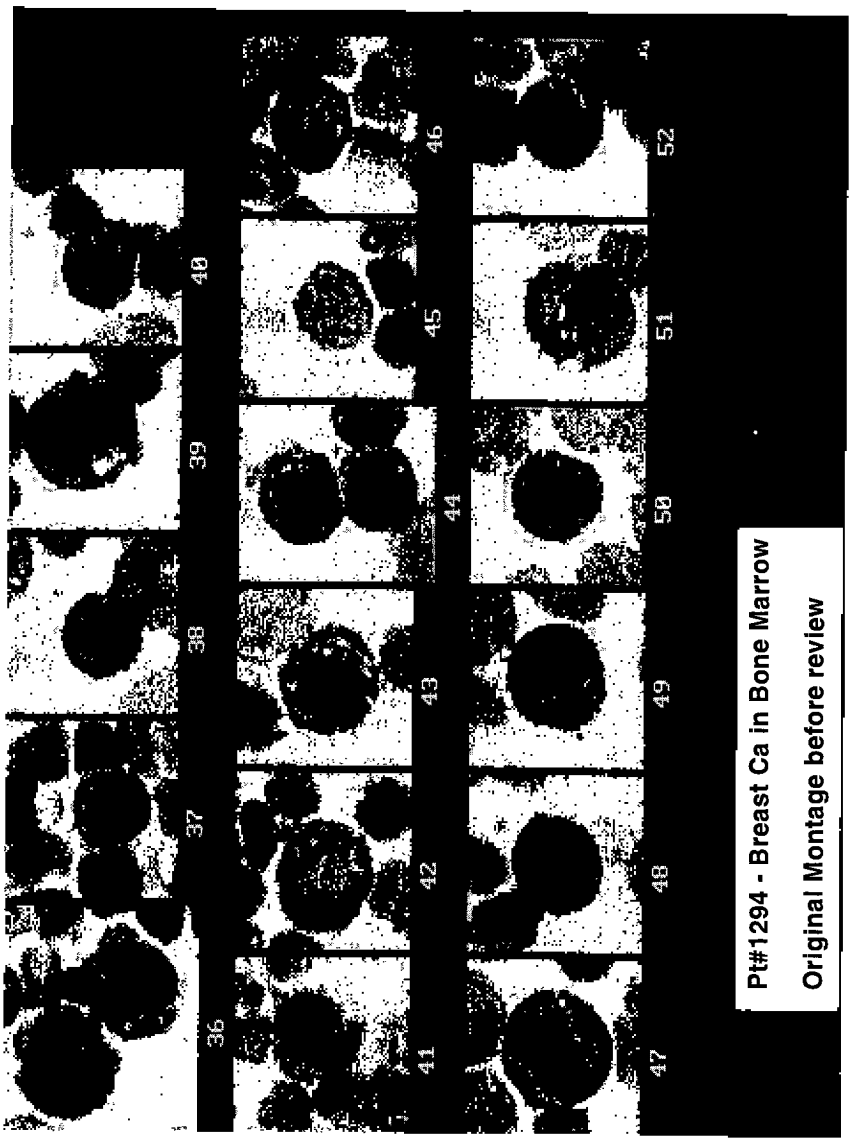

FIGURE A2.2. At the completion of the run, the reviewer is presented with a visual montage of all of the events detected by the instrument.

should also be applicable to the detection of early recurrent cancer. The technical problem with bringing this analysis into the routine clinical workup is that it requires special stains to enhance detection and tedious and careful microscopic examination of the slides (by highly trained staff) to identify these rare events.

Detection of rare tumor cells in BM is only the initial step. The presence of these cells does not necessarily mean that they have the properties necessary to form fully malignant metastatic tumors. Although there is a strong correlation in all tumor types studied between the presence of such cells and outcome, the correlation is not perfect. Therefore, reagents and protocols are necessary to characterize the cells by interrogating them to determine whether they possess all of the characteristics of fully malignant cells. The use of in situ amplification techniques and multiple color staining will enable us to determine if the detected cells are capable of proliferation, invasiveness, metastasis, and angiogenesis. It will enable us to look for the activation of cellular oncogenes and for the mutation or deletion of suppressor genes.

The presence or absence of fully malignant tumor cells in BM will enable the oncologist to aggressively treat patients who need therapy as well as to modify treatment (eg, prostate cancer patients with BM tumor cells would be treated systemically instead of having a prostatectomy, with all of its sequelae) and to not treat patients who do not need therapy or whose disease has progressed to the point where therapy will no longer be curative. Detection and density of antigens on the surface of the detected tumor cells can be used as criterion for guiding therapy, for appropriate patients, into the realm of passive or active immunotherapy rather than classical chemotherapy. Detection of drug resistance markers on the tumor cells will also prevent the use of inappropriate (ineffective) chemotherapy with all of its severe side effects. Other applications in this area are also under investigation. The net effect, will be to improve therapy and tailor it to individual patients in such a manner that outcomes will improve and costs will lower.

DETECTION OF RARE EVENTS IN INFECTIOUS DISEASES

In situ PCR and RT–PCR techniques are rapidly becoming established techniques for detection of intracellular products. The number of poten-

tial research and diagnostic applications continues to grow (5, 6). In particular, the detection of viral and proviral gene sequences has found significant utility in diagnosis and prognostic applications. While traditional in situ hybridization techniques using polyclonal and monoclonal antibody detection have been used for a number of years to detect virally infected cells, the ability to quantitate these so-called rare events (cells expressing viral proteins) has not achieved significant clinical utility. With PCR and RT–PCR, there is improved sensitivity, specificity, and the capacity to identify multiple nucleic acid targets within individual cells. These technologies are expected to accelerate the development of improved methods with increased diagnostic accuracy and reliability in viral diseases. Bagasra and coworkers (7) have reported successful amplification and detection of human immunodeficiency virus (HIV-1), simian immunodeficiency virus (SIV), hepatitis B virus (HBV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpes virus type 6 (HHV-6), herpes simplex virus (HSV), and lymphogranuloma venereum (LGV). In addition, in situ PCR and RT–PCR have been applied to studies of endogenous gene sequences and the detection of low copy number mRNA. These exciting breakthroughs allow the possibility for analysis of cells with specialized probes detecting markers of interest in vaccine development and gene therapy, transgenic animal studies, bacterial and viral drug resistance, and cellular proteins and receptor expression (8).

One of the remaining limitations in the clinical use of in situ PCR and RT–PCR for detection of rare cells in infectious diseases is the need to manually examine microscope slides with large numbers of cells to detect virally infected cells present at low frequency, which is tedious and inefficient. As molecular probe technology has improved, it has become increasingly necessary to automate the final step in the procedure, the detection, quantitation, and image capture of target positive events (9). Recent improvements in automating microscopic analysis of bright field and fluorescent cell preparations now allow rapid scanning and image capture of larger numbers of cells than previously possible with manual review of slides. As previously described for tumor cells, it is now possible in just a few minutes for an automated system to determine optimal focus, review hundreds of thousands of cells on a single slide at more than one magnification, and to capture images of virally infected cells of interest. The images are then saved for operator review. Software programs can be used to calculate the number of cells infected per 10^5 cells counted for each slide. This allows quantitative analysis of rare events such as the

number of infected cells per 100,000 cells as a measure of viral burden (10). With multianalyte probes or the use of both nucleic acid probes and monoclonal antibodies, quantitation of infected cells within a subset of the total cells reviewed is possible. An important infectious disease application is the quantitation of the number of CD4 bearing T-lymphocytes containing either replicating (RNA) or integrated (proviral DNA) HIV-1 (7). The quantitation of viral replication can have significant clinical implications and is discussed in more detail in the following sections.

Quantitation of HIV-1 Viral Burden

HIV disease progression and the development of drug resistance are known to be associated with viral load in the infected individual (11, 12). Single or low copy number proviral DNA can be quantitated using in situ hybridization by PCR methods and detected by flow cytometric methods (13), titration (14), and microscopy (15). In addition, methods for detection of HIV-1 RNA-producing cells have been developed to monitor HIV replication and cell-associated viral load (7, 16, 17). The relationship between cell-associated, infectious HIV-1 and cell-free virus load is currently under active investigation. Current methods for quantitating HIV-1 RNA in plasma by various nucleic acid probe amplification procedures suffer from technological limitations involving sensitivity, false amplification due to contamination, and complexity in addition to high cost. In situ hybridization methods may prove to be less costly and technically comparable. It is expected that accurate quantitation of HIV-1 viral load will be clinically useful in staging the risk of HIV-1 disease progression and in monitoring antiviral drug efficacy.

Automated Microscopic Detection of HIV-1 using RT–PCR and in situ Hybridization

Using a high speed, automated microscopy system, the feasibility of rapid quantitation of HIV-1 RNA-containing cells detected by in situ hybridization using both RT–PCR and synthetic oligo proves has been shown in our laboratory (15). The use of colored precipitating substrates permits rapid detection of rare HIV-1 RNA-expressing cells as described earlier for tumor cell detection. The practical detection limit of one cell in 10^6 to10^8 is well within the range necessary for detection of HIV-1-infected cells with little or no enrichment of the CD4+ cells necessary.

Standardization of procedures and the availability of commercial reagents will allow definitive studies of the utility of quantitation of cell-associated HIV-1 in clinical decision making.

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Contributed by
Timothy J. Holzer and Herbert Lazarus
Diagnostics Division, Centocor, Inc.
Malvern, PA 19355–1307

INDEX

ABOUT THE AUTHORS

Omar Bagasra, M.D., PhD.

In 1948, somewhere on the plains of India, Omar Bagasra was born in the back of a wooden oxcart. His refugee family was migrating north during the exodus of the 25 million souls who were forced to leave their ancestral homelands when the former British colony of India was being partitioned during its struggle to become independent. At least 8 million of these refugees—Sikhs, Muslims, and Hindus alike—perished in this partitioning. Being Muslim, Omar's family settled in the new nation of Pakistan, where his father became a successful grain merchant and where 10 more brothers and sisters were born and 1 was adopted. In this somewhat volatile environment, Omar grew into a young man.

At the outbreak of the India–Pakistan War of 1965, Omar volunteered for the Pakistani Army when he was just 16 years old. His interest in premedical studies quickly led him to be trained as a medical corpsman, and his unit was thrust into the especially vicious Battle of Sialkot. Omar survived this quite sanguinary engagement unscathed, but many other combatants did not.

After this particularly harrowing experience, Omar decided to study Buddhism, a religion for which warfare is anathema. He left his parents' home in Pakistan and journeyed to a monastery in Tibet. After two years of Lamaistic study and contemplation, Omar decided that the scientific

understanding of nature was just as important a path to truth as the more mystical, consciousness approach of the monks. Omar describes his experience this way, "I always liked the study of science, but I am very happy I got the chance to study a second religion as well. This helped me better appreciate Islam and its rich history as well as the analytic methods of science. Facts and logic are quite important to Islam, actually; and Muslim culture has contributed a great deal to the study of astronomy, medicine, and mathematics—particularly in those formative years of science before the European Renaissance. Studying Buddhism helped me realize this."

He, therefore, returned to Pakistan and enrolled in the University of Karachi, where he earned a bachelor's degree and a master's degree in biochemistry. "I wanted to get even higher education," he says, "but in Pakistan at that time, that was as high as I could get." So in 1972 he flew to Chicago's O'Hare Airport, carrying just a suitcase of clothing and an extra 100 dollars in his pocket.

Omar did not know anyone in the United States, but he soon found employment in the road construction industry, and he improved his English—his seventh language. He then got a better job manufacturing brake shoes for the Ford Motor Company near Fort Wayne, Indiana. Omar saved his wages and enrolled at the University of Louisville; soon he got his first scientific job working as a lab tech at the nearby Clark County Memorial Hospital. There he met a young nurse, Theresa Mahoney, and the two were married. By 1980, Omar had earned a Ph.D. in microbiology and immunology and the family moved to Philadelphia, where Omar became a junior faculty member at Hahnemann University and a citizen of the United States.

Soon thereafter, Omar decided to go to medical school. But admissions policies at that time were restrictive for individuals born and educated overseas, so the 32-year-old Omar—never one to be confined by national borders—went to study medicine at the Universidad Autónoma, Ciudad Juaréz, Mexico. After two years of study, he went to Temple University, where he completed his clinical training.

He soon rose to another faculty position at Hahnemann University. He next went to the University of Medicine and Dentistry of New Jersey, had a brief stint at St. Christopher's Hospital for Children in Philadelphia. Finally, he moved to Thomas Jefferson University in Philadelphia, where he serves as director of the Molecular Retrovirol-

ogy Laboratories, section chief of molecular diagnostics of the Center for the Study of Human Viruses, and associate professor of infectious diseases. Omar also keeps a hand in clinical work; he is currently board-eligible in anatomic pathology and a diplomate of the American Board of Medical Laboratory Immunology. He is also board-certified in forensic pathology.

Omar's research interests have long been associated with the study of HIV and AIDS. In fact, he has been on the trail of the virus since 1981—the year of the first scientific report. For the past several years, Omar has focused on trying to gain insight into modes of virus transmission, natural immunities to retroviral infection, and the development of antisense and gene therapy treatments for HIV-1. His unswerving dedication to his work has resulted in more than 150 scientific articles and book chapters, and he frequently serves as an invited speaker at scientific conferences throughout the world.

Omar and Theresa now have two children, Alexander (17 years) and Anisah (15 years), and the family resides in Laurel Springs, New Jersey.

John Hansen

John Hansen grew up on a farm in southern Maryland, among the headwaters of the Zekiah Swamp. At the age of 12, he went to study at a Benedictine monastery school in Washington, D.C., paying his tuition through the culture of strawberries and tobacco. In 1978, with the opportunity of financial aid, he was able to matriculate to Harvard College in Cambridge, Massachusetts.

Following his commencement, John went to work in the U.S. House of Representatives on Capitol Hill. At the age of 26, he ran for election, to represent Charles County in the Maryland House of Delegates. With support from labor and environmental groups, he narrowly missed defeating a 12-year incumbent. Soon thereafter, John's long-time mentor, the Reverend Michael Peterson, M.D., succumbed to the ravages of AIDS. Now without a job or a teacher, John returned to Massachusetts and to much-needed employment at the Harvard University Library and graduate studies in atmospheric chemistry and North American archaeology.

About this time, two college friends were founding a company to manufacture equipment for molecular biology laboratories. John helped out in the early days and then went to work full-time as director of special

projects; he currently serves as vice president for marketing and communications. Today, the company MJ Research—is the world's leading manufacturer of Peltier thermal cyclers, with distributors in more than 40 nations and whose instruments are in use on all seven continents of the earth.

John currently resides in Central Square, Cambridge, Massachusetts.

Color Figure Section

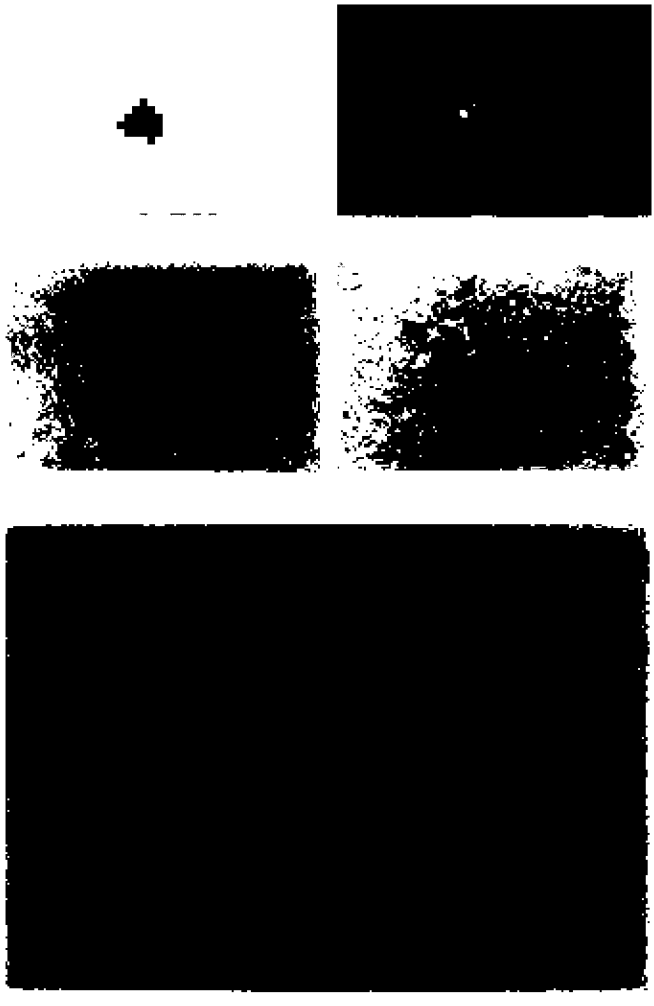

FIGURE 3. DNA and RNA in situ PCR at the single-cell level. Top panel: dual amplification of DNA and multiply spliced mRNA in a single cell. Upper left: in situ DNA PCR (red nuclear signals); upper right: in situ RT–PCR for mRNA HLADQa as observed by FITC-labeled probe. Note the nuclear versus cytoplasmic staining. Middle panel: in situ RT–PCR of HIV-1 in oral mucosal epthelial cells of an HIV-1 seropositive individual. Left: in situ RT–PCR for HIV-1 seronegative specimen. Right: in situ RT–PCR for HIV-1 in a specimen from HIV-1 infected person. Note the strong trapping of signals and distinct loci in the infected cells and the lack of diffuse or background signals. For the detection of HIV-1 RNA, *tat* multiply spliced mRNA sequences were used. Lower panel: in situ PCR on the chromosome bands. DNA in situ PCR was performed on the chromosomal bands from a SUP-T1 cell line infected with HIV-1. *Arrows* show the points of insertion of HIV-1.

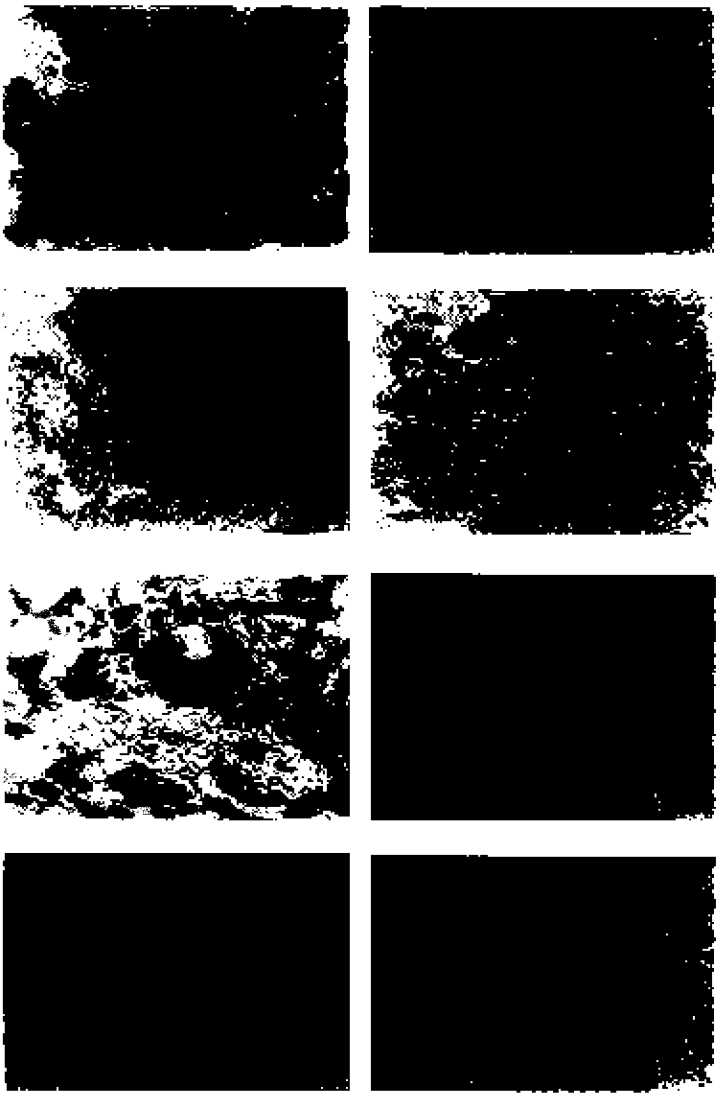

FIGURE 4. DNA in situ PCR for human herpesvirus type 8 (HHV-8). Upper panel: high power view of a Kaposi's sarcoma lesion; many cells show positive staining (red cells) for HHV-8. Middle panel: (left) low power view of a tissue section from skin from a healthy person, subjected to in situ PCR for HHV-8, no positive signal obtained. (Right) a tissue section from an AIDS-associated Kaposi's sarcoma lesion. Note HHV-8 positive signal in a nodular lesion. Third panel: (left) high power view of Kaposi's sarcoma lesion in an AIDS patient. Note the infection of the microvascular endothelial cells. (Right) HHV-8 in the sperm specimen from a healthy person, absence of HHV-8. Lower panel: (left-right) presence of HHV-8 in the sperm heads of two semen specimens from the AIDS patient.

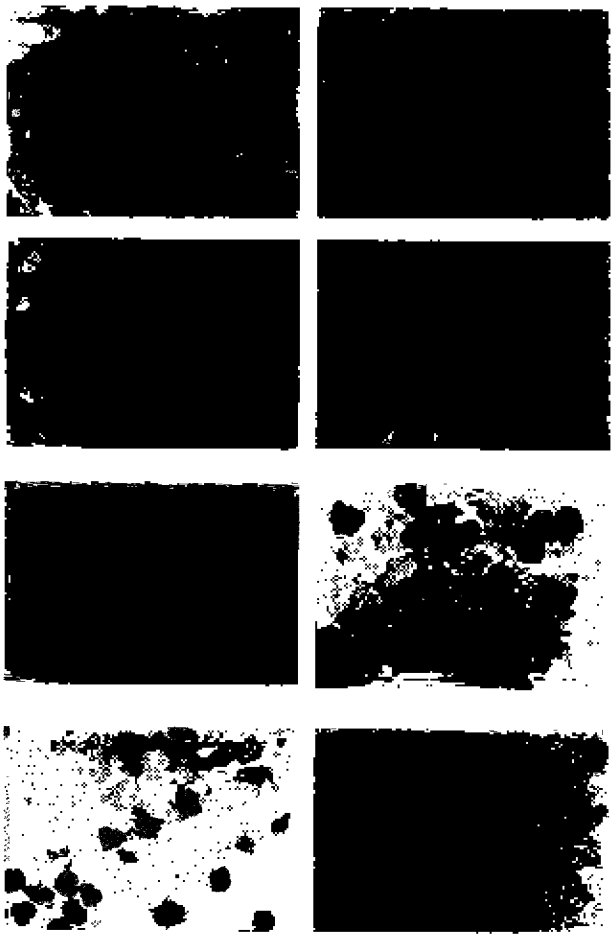

FIGURE 5. DNA in situ PCR for HHV-8 and their association with Kaposi's sarcoma. Upper right panel: high power view of two non-AIDS Kaposi's sarcoma lesions; many cells show positive staining (red cells) for HHV-8, including the microvascular endothelial cells. Middle panel: (left) high power view of a non-AIDS Kaposi's sarcoma lesion from an endemic African area; many cells show positive staining (red cells) for HHV-8, including the microvascular endothelial cells. (Right) presence of episomal HHV-8 in a non-AIDS Kaposi's sarcoma lesion. Third panel: (left) presence of episomal HHV-8 in an AIDS-Kaposi's sarcoma lesion. (Right) in situ PCR of the PBMC from a patient with AIDS-related Kaposi's sarcoma. These studies used an HHV-8 primer pair for in situ PCR and subsequently a biotinylated HHV-8 probe from the 330 Bam sequence. The *arrow* indicates a cell with a positive signal. Lower panel: (left) PBMC from a normal healthy person. (Right) in situ PCR performed on T and B lymphocyte subpopulations from the same person as above. Positive signals were seen in both subsets, more in B cells than in T cells. The B cell fraction is shown here.

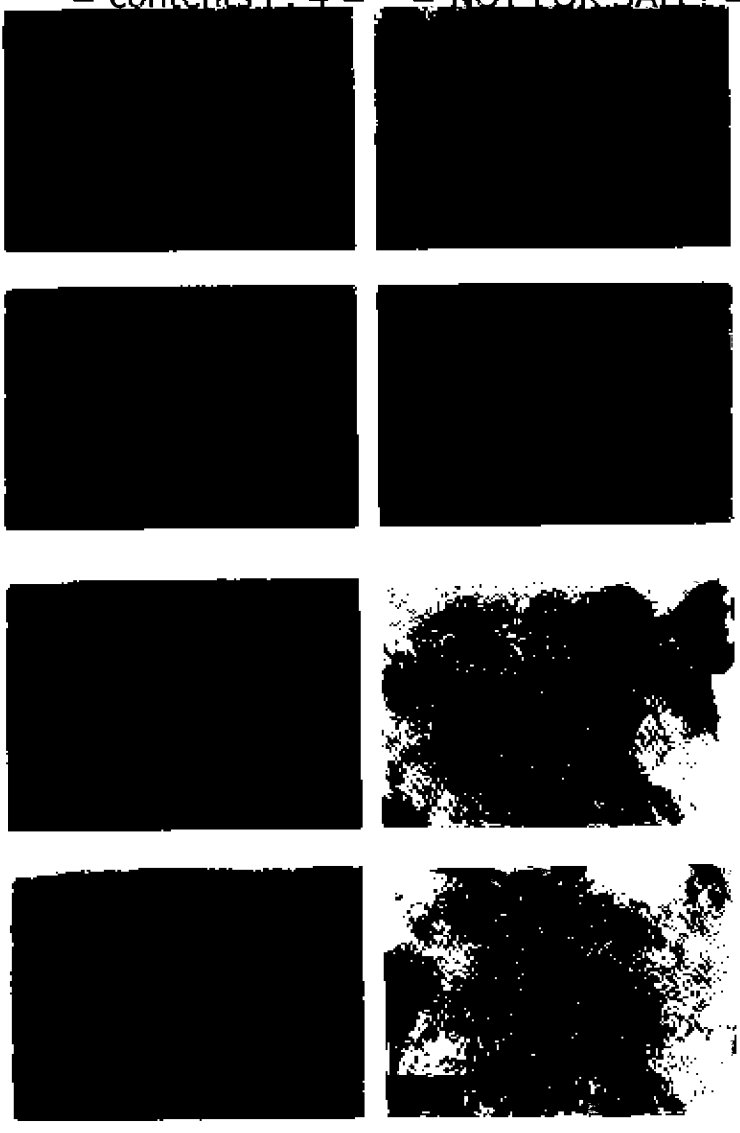

FIGURE 6. In situ PCR on primary brain cell cultures infected with HIV-1. Upper panel: (left) infection of microvascular endothelial cells with HIV-1. (Right) control, uninfected cells. Middle panel: (left) infection of astrocytes with HIV-1. (Right) a cell line derived from human microvascular endothelial cells infected with HHV-8. Note the episomal nature of the virus. Third panel: (left) a different cell line derived from human microvascular endothelial cells infected with HHV-8. Again note the episomal nature of the virus. (Right) HHV-8 in the brain tissues of HIV-1 infected patients. Lower panel: (left-right) HHV-8 in the brain tissues of HIV-1 infected patients with severe dementia.

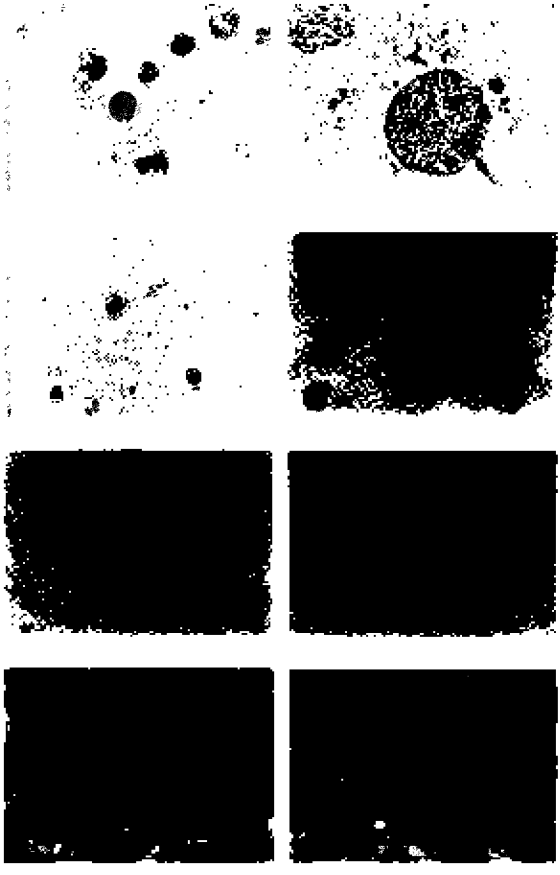

FIGURE 7. In situ PCR for transgenic plants. Upper panel: (left) as a control, tomato protoplasts were subjected to in situ PCR for the large subunit of the endogenous plant gene 1,5-biphosphate carbooxylase. (Right) control for the same gene, without primers. Middle panel: (left) control for transgenic rabies gene. (Right) in situ PCR for rabies G-protein gene. Third panel: as an additional control. tomato protoplasts were subjected to in situ PCR for the small subunit of the endogenous plant gene 1,5-biphosphate carbooxylase. (Right) control for the same gene, without primers. Lower panel: (left) an MS-plaque area exhibiting mRNA expression of inducible nitric oxide synthase–positive round cells (green), as determined by reverse transcriptase initiated in situ PCR. Note the severe destruction of neuronal filaments (red), detected using a cocktail of three monoclonal antineurofilament antibodies (marker for neurofilaments). None of the cells positive for iNOS reacted with neurons. (Right): the same MS-plaque area exhibiting mRNA expression of inducible nitric oxide synthase–positive round cells (green), as determined by RT in situ PCR. Note the severe destruction of astrocytic processes (red), detected using a monoclonal antigilal fibrillary acidic protein antibody (marker for astrocyte). None of the cells positive for iNOS reacted with astrocytes.

IN SITU PCR

TECHNIQUES

OMAR BAGASRA
JOHN HANSEN

In situ gene amplification techniques offer tremendous potential as aids to clinical diagnosis through their ability to detect a single copy of a specific microbial, neoplastic, messenger, or mutated nucleic acid sequence in a cell smear, cell suspension, tissue section, or chromosome. In situ hybridization, applied in combination with the polymerase chain reaction (PCR), can be expected to fuel accelerated developments in the understanding of embryogenesis, organogenesis, and cell differentiation, as well as the pathogenesis of numerous disease processes. But the procedures are cumbersome and fraught with potential variables, and experimental results are difficult to reproduce.

In Situ PCR Techniques addresses this problem directly, with comprehensive step-by-step protocols for the delineation of genetic amplification and histological detection techniques. Each procedure has been tested and validated for its sensitivity, precision, and reproducibility, and the authors give advice on the design of primers for PCR applications and on optimizing these protocols for use with plant, insect, and prokaryotic cells. They facilitate the repetition of published experiments by providing the kinds of hints, tips, and laboratory secrets that are often left out of scientific papers. They also demonstrate clearly and thoroughly the efficacy of in situ PCR as an alternative to standard and fluorescence in situ hybridization for detecting a single copy of a mutant gene, a virus, or a very low-abundance message in individual cells, while preserving the morphology of the cell and the tissue in which it resides.

Topics covered include:

- A review of the in situ PCR technique
- Step-by-step protocols for in situ PCR techniques
- Optimization of annealing temperatures for specific primers
- Preparation of glass slides and tissues
- Selection of DNA and RNA targets
- Combination of immunohistochemistry and in situ PCR amplification
- Special applications of in situ PCR amplification
- Hybridization reactions
- Validation and controls
- Materials and methods

Supplemented with numerous helpful graphics and illustrations, *In Situ PCR Techniques* is immensely useful, not merely to the novice, but also to experienced researchers in investigative pathology, virology, and gene therapy, as well as in developmental biology, immunology, plant molecular biology, entomology, and the neurosciences.

Cover Design: Roberta Ludlow

Cover illustration: An MS-plaque area exhibiting mRNA expression of inducible nitric oxide synthase (iNOS)-positive round cells (green), as determined by reverse-transcriptase-initiated (RT)-in situ polymerase chain reaction and severe destruction of neuronal filaments (red), detected using a cocktail of three monoclonal anti-neurofilament antibodies, a marker for neurofilaments.

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