1

Introduction to the Polymerase Chain Reaction

Y. M. Dennis Lo

1. Introduction

The polymerase chain reaction (PCR) is an in vitro method for the amplification of DNA that was introduced in 1985 *(1)*. The principle of the PCR is elegantly simple but the resulting method is extremely powerful. The adoption of the thermostable *Taq* polymerase in 1988 greatly simplifies the process and enables the automation of PCR *(2)*. Since then a large number of applications have been developed that are based on the basic PCR theme. The versatility and speed of PCR have revolutionized molecular diagnostics, allowing the realization of a number of applications that were impossible in the pre-PCR era. This chapter offers an introductory guide to the process.

2. Principle of the PCR

PCR may be regarded as a simplified version of the DNA replication process that occurs during cell division. Basic PCR consists of three steps: thermal denaturation of the target DNA, primer annealing of synthetic oligonucleotide primers, and extension of the annealed primers by a DNA polymerase (**Fig. 1**). This three step cycle is then repeated a number of times, each time approximately doubling the number of product molecules. The amplification factor is given by the equation $n(1 + E)^x$ where n = initial amount of target, E = efficiency of amplification, and x = number of PCR cycles. After a few cycles, the resulting product is of the size determined by the distance between the 5'-ends of the two primers. With the performance of a previous reverse transcription step, PCR can also be applied to RNA (*see* Chapter 14).

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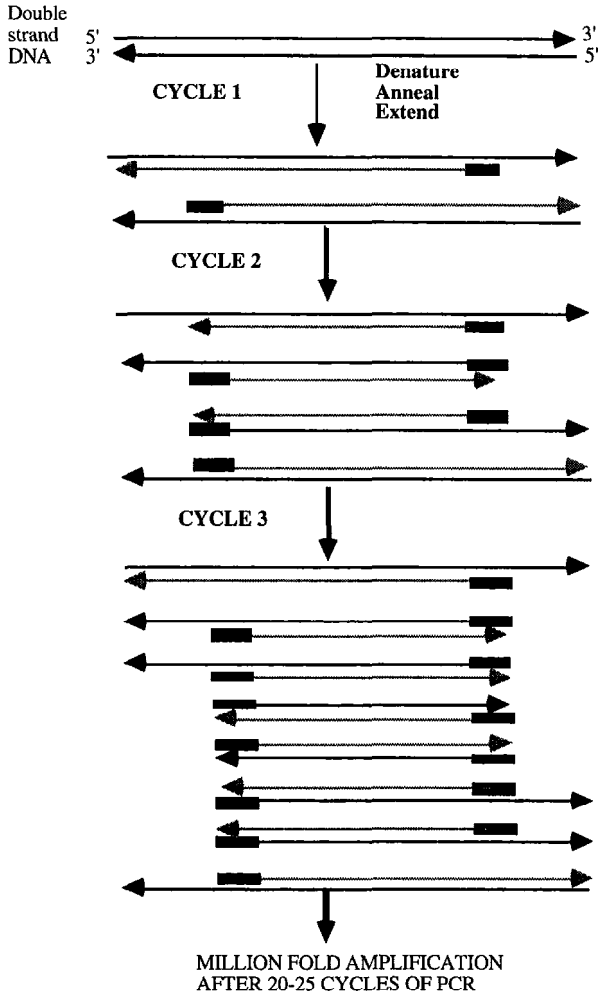

Fig. 1. Schematic representation of the polymerase chain reaction. The newly synthesized DNA is indicated by dotted lines in each cycle. Oligonucleotide primers are indicated by solid rectangles. Each DNA strand is marked with an arrow indicating the 5' to 3' orientation.

3. Composition of the PCR

PCR is usually performed in a volume of 25–100 µL. Deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) at a concentration of 200 µM each, 10 to 100 pmol of each primer, the appropriate salts, buffers, and DNA polymerase are included. Many manufacturers have included reaction buffer with their DNA polymerase and this practice is convenient to newcomers to the PCR process.

4. Primers

Primers are designed to flank the sequence of interest. Oligonucleotide primers are usually between 18 and 30 bases long, with a GC content of about 50%. Complementarity at the 3'-ends of the primers should be avoided to decrease the likelihood of forming the primer–dimer artifact. Runs of three or more C's or G's at the 3'-ends of the primers should be avoided to decrease the probability of priming GC-rich sequences nonspecifically. A number of computer programs are available to assist primer design. However, for most applications PCR is sufficiently forgiving in that most primer pairs seem to work. The primers are generally positioned between 100 to 1000 bp apart. It should be noted, however, that for high sensitivity applications, shorter PCR products are preferred. For most applications, purification of the PCR primers are not necessary. To simplify subsequent operations, it is recommended that all primers are diluted to the same concentration (e.g., 50 pmol/μL) such that the same volume of each primer is required for each reaction. Some primer pairs seem to fail without any obvious reason, and when difficulty arises, one simple solution is to change one or both of the primers.

The use of primers for allelic discrimination (Chapters 7 and 8) and the application of labeled primers (Chapters 6, 20, and 23) are described later on in the book.

5. Steps of the PCR

5.1. Thermal Denaturation

A common cause of failed PCR is inadequate denaturation of the DNA target. We typically use an initial denaturation temperature of 94°C for 8 min. For subsequent cycles, 94°C for 1–2 min is usually adequate. As the targets of later PCR cycles are mainly PCR products rather than genomic DNA, it has been suggested that the denaturation temperature may be lowered after the first 10 cycles so as to avoid excessive thermal denaturation of the *Taq* polymerase *(3)*. The half-life of *Taq* DNA polymerase activity is more than 2 h at 92.5°C, 40 min at 95°C and 5 min at 97.5°C.

5.2. Primer Annealing

The temperature and length of time required for primer annealing depends on the base composition and the length and concentration of the primers. Using primers of 18–30 bases long with approx 50% GC content, and an annealing step of 55°C for 1–2 min is a good start. In certain primer–template pairs, a small difference in the annealing temperature of 1–2°C will make the difference between specific and nonspecific amplification. If the annealing temperature is >60°C, it is possible to combine the annealing and extension step together into a two step PCR cycle.

5.3. Primer Extension

Primer extension is typically carried out at 72°C, which is close to the temperature optimum of the *Taq* polymerase. An extension time of 1 min is generally enough for products up to 2 kb in length. Longer extension times (e.g., 3 min) may be helpful in the first few cycles for amplifying a low copy number target or at later cycles, when product concentration exceeds enzyme concentration.

6. Cycle Number

The number of cycles needed is dependent upon the copy number of the target. As a rule of thumb, to amplify 10^5 template molecules to a signal visible on an ethidium bromide stained agarose gel, requires 25 cycles. Assuming that we use 1 min each for denaturation, annealing and extension, the whole process can be completed in approx 2–3 h (with extra time allowed for the lag phase taken by the heat block to reach a certain temperature). Similarly, 10^4, 10^3, and 10^2 target molecules will require 30, 35, and 40 cycles, respectively. Careful optimization of the cycle number is necessary for quantitative applications of PCR (*see* Chapter 4).

7. PCR Plateau

There is a limit to how many product molecules a given PCR can produce. For a 100 µL PCR, the plateau is about 3–5 pmol ***(4)***. The plateau effect is caused by the accumulation of product molecules that result in a significant degree of annealing between complementary product strands, rather than between the primers and template. Furthermore, the finite amount of enzyme molecules present will be unable to extend all the primer–template complex in the given extension time.

8. Sensitivity

The sensitivity of PCR is related to the number of target molecules, the complexity of nontarget molecules, and the number of PCR cycles. Since the introduction of the *Taq* polymerase, it has been known that PCR is capable of amplification from a single target molecule ***(2,5)***. This single-molecule capability has allowed the development of single sperm typing ***(5,6)*** and preimplantation diagnosis ***(7–9)*** (*see* Chapters 20 and 22). In these applications, the single target molecule is bathed, essentially, in PCR buffer—in other words, in a low complexity environment. In situations where the complexity of the environment is high, the reliability of single molecule PCR decreases and strategies such as nesting and Hot Start PCR ***(10,11)*** are necessary for achieving maximum sensitivity (*see* Chapters 11, 15, 18, 19, and 21). The sensitivity of PCR has also allowed it to be used in situations where the starting materials have been partially degraded (*see* Chapter 3).

9. PCR Fidelity

The fidelity of amplification by PCR is dependent upon several factors: annealing/extension time, annealing temperature, dNTP concentration, $MgCl_2$ concentration, and the type of DNA polymerase used. In general, the rate of misincorporation may be reduced by minimizing the annealing/extension time, maximizing the annealing temperature, and minimizing the dNTP and $MgCl_2$ concentration ***(12)***. Eckert and Kunkel reported an error rate per nucleotide polymerized at 70°C of 10^{-5} for base substitution and 10^{-6} for frameshift errors under optimized conditions ***(12)***. The use of a DNA polymerase with proofreading activity reduces the rate of misincorporation. For example, the DNA polymerase from *Thermococcus litoralis*, which has proofreading activity, misincorporates at 25% of the rate of the *Taq* polymerase, which lacks such activity ***(13)***. Interestingly, the combination of enzymes with and without proofreading activity has enabled the amplification of extremely long PCR products (*see* Chapter 9).

For most applications, product molecules from individual PCR are analyzed as a whole population and rare misincorporated nucleotides in a small proportion of molecules pose little danger to the interpretation of data. However, for sequence analysis of cloned PCR products, errors due to misincorporation may sometimes complicate data interpretation. Thus, it is advisable to analyze multiple clones from a single PCR or to clone PCR products from several independent amplifications. Another application where misincorporation may result in error in interpretation is in the amplification of low copy number targets (e.g., single molecule PCR). In these situations, if a misincorporation happens in an early PCR cycle (the extreme case being in cycle 1), the error will be passed onto a significant proportion of the final PCR products. Hence, in these applications, the amplification conditions should be carefully optimized.

10. PCR Thermocyclers

One of the main attractions of PCR is its ability to be automated. A number of thermocyclers are available from different manufacturers. These thermocyclers differ in the design of the cooling systems, tube capacity, number of heating blocks, program memory, and thermal uniformity. In our opinion, units using the Peltier system are fast and have a uniform thermal profile across the block. Units with multiple heating blocks are very valuable for arriving at the optimal cycling profile for a new set of primers, as multiple conditions can be tested simultaneously. Tube capacity generally ranges from 48 to 96 wells and should be chosen with the throughput of the laboratory in mind. Some thermocyclers have heated covers and, thus, allow the omission of mineral oil from the reaction tubes. Specially designed thermal cyclers are required for *in situ* amplification (*see* Chapter 12) that accommodate glass slides.

11. Analysis and Processing of PCR Product

The amplification factor produced by PCR simplifies the analysis and detection of the amplification products. In general, analytical methods for conventional DNA sources are also applicable to PCR products. Some of these methods for studying sequence variation are covered in this volume (*see* Chapters 5, 6, and 13)

11.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis followed by ethidium bromide staining represents the most common way to analyze PCR products. A 1.5% agarose gel is adequate for the analysis of PCR products from 150 to 1000 bp. A convenient molecular weight marker for this size range is ΦX174 DNA digested by *Hae*III.

11.2. Restriction of PCR Products

Restriction mapping is a commonly used way of verifying the identity of a PCR product. It is also a simple method of detecting restriction site polymorphisms and for detecting mutations that are associated with the creation or destruction of restriction sites. There is no need to purify the PCR product prior to restriction and most restriction enzymes are functional in a restriction mix in which the PCR product constitutes up to half the total volume.

11.3. Sequence-Specific Oligonucleotide Hybridization

This is a powerful method for detecting the presence of sequence polymorphisms in a region amplified by PCR. Short oligonucleotides are synthesized and labeled (either radioactively or nonradioactively), allowed to hybridize to dot blots of the PCR products *(5)*, and washed under conditions that allow the discrimination of a single nucleotide mismatch between the probe and the target PCR product.

For the detection of a range of DNA polymorphisms at a given locus, the hybridization can be performed "in reverse," that is, with the oligonucleotides immobilized onto the filter. Labeled amplified products from target DNA are then hybridized to the filters and washed under appropriate conditions *(14)*. The reverse dot-blot format is now available for many multi-allelic systems *(15,16)*.

11.4. Cloning of PCR Product

PCR products may be cloned easily using conventional recombinant DNA technology. To facilitate cloning of PCR products into vectors, restriction sites may be incorporated into the primer sequences. Digestion of the PCR products with the appropriate restriction enzymes will then allow "sticky end" ligation into similarly restricted vector DNA.

12. Conclusion

The versatility of PCR has made it one of the most widely used methods in molecular diagnosis. The number of PCR-based applications have continued to increase rapidly and have impacted in oncology (*see* Chapters 15 and 17–19), genetics (*see* Chapters 16 and 20–23), and microbiology (*see* Chapters 24 and 25). In this book we attempt to present some of the most important clinical applications of PCR.

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3

Amplification from Archival Materials

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1. Introduction

The ability of the polymerase chain reaction (PCR) to amplify from partially degraded and relatively impure preparations has allowed the technique to amplify nucleic acid sequences from archival materials, which in many institutions consist of paraffin-embedded tissue samples *(1,2)*. This ability has allowed the carrying out of large scale retrospective studies of archival materials and has facilitated the use of materials from multiple institutions from different countries *(3)*.

The preparation of paraffin embedded tissues for PCR analysis involves a number of steps. The first is the dewaxing of paraffin from the tissue samples. This is then followed by procedures designed to liberate the DNA from the samples. A variety of techniques have been used that include boiling *(4)*, proteinase K digestion *(5)* and treatment with Chelex 100 *(6,7)*. In many situations, complete nucleic acid purification is unnecessary and indeed undesirable because the additional steps involved may increase the risk of contamination. The dewaxing and DNA liberation steps are then followed by PCR amplification.

The success of PCR from paraffin-embedded materials depends, to a large extent, on the fixation of the samples *(8,9)*. Fixation parameters that have been found to be important include:

1. The type of fixative: the best fixatives for preserving materials for subsequent PCR are ethanol, acetone, Omnifix, and 10% neutral buffered formalin (NBF) *(9)*
2. Duration of fixation: generally extended fixation time is detrimental to PCR analysis of the materials *(8)*. Furthermore, longer PCR targets appear to be more sensitive to the effect of prolonged fixation than shorter PCR targets.

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3. Specimen age: generally, the older the specimen age, the less amenable it is for PCR amplification. This is especially the case for long amplicons. Thus, it is recommended that shorter length amplicons be used for old paraffin-embedded samples

2. Materials

2.1. Sample Processing

1. Paraffin-embedded tissue sections
2. Xylene (HPLC grade) (Aldrich, Milwaukee, WI)
3. 95% Ethanol.
4. Proteinase K (20 mg/mL stock solution) (Boehringer Mannheim, Sussex, UK).
5. Proteinase K digestion buffer: 50 m*M* Tris-HCl, pH 8 5, 1 m*M* EDTA, 0 5% Tween-20 (Sigma, Poole, UK).
6. 10% bleach solution (freshly made daily)
7. Eppendorf tubes.
8 Disposable microtome blades (*see* **Note 1**).
9. Microtome.
10. Oven
11. Vortex mixer.
12. Sterile Pasteur pipets
13. Microcentrifuge.

2.2. PCR

1. Thermal cycler.
2. PCR buffer II and magnesium chloride solution (Perkin Elmer, Norwalk, CT).
3. dNTPs (Perkin Elmer)
4. *Taq* DNA polymerase (Perkin Elmer).
5. Primers (Genosys)· typically 10–100 pmol per 100 µl reaction.

3. Methods

3.1. Cutting of Paraffin-Embedded Sections

1. Use a microtome to cut paraffin embedded sections from tissue samples. Push the cut sections into a recipient Eppendorf tube using a sterile Pasteur pipet.
2. Employ a new disposable blade with each sample. Clean the microtome carefully with 10% bleach following each specimen (*see* **Notes 1–4**)

3.2. Preparation of Cut Sections

1. Deparaffinize sections by adding 400 µL xylene, vortexing for 1 min, and spinning for 5 min in a microcentrifuge.
2. Pipet off the xylene carefully.
3. Add 400 µL of 95% ethanol. Vortex for 1 min and spin for 5 min.
4. Pipet off most of the ethanol.
5. Repeat **steps 3** and **4**.
6. Remove most of the ethanol.

7. Incubate the tube with their caps open in an 80°C oven for 10 min (*see* **Note 5**).
8. Add 100 µL of proteinase K digestion buffer with 200 mg/mL proteinase K to each tube.
9. Digest from 3 h to overnight at 37°C.
10. Following digestion, spin the tube briefly and heat at 94°C for 20 min to inactivate the proteinase K (*see* **Notes 6** and **7**).
11. Use 10 µL for PCR (*see* **Notes 8** and **9**).

4. Notes

1. Obsessive care should be taken to prevent cross contamination between samples. As it is difficult to completely clean cutting surfaces, disposable blades are the ideal option. Guidelines on minimizing the risk of contamination are described in Chapter 2.
2. Always discard the first slice from a paraffin wax embedded block. Subsequent slices can be regarded as "clean," being "protected" from the environment by the first slice.
3. The thickness and the number of slices taken for preparation depends on the specimen size. As a guideline, 1–10, 5 µm sections are cut from liver resection specimens averaging 2 cm^2 in surface area.
4. If the area of interest is small, it may be advisable to use the first and last slices for hematoxylin and eosin (H&E) staining to ascertain that the area of interest has indeed been cut.
5. The open tubes are especially prone to contamination at this point.
6. Note that the protocol described here does not involve further DNA purification using phenol/chloroform extraction. This is to minimize the number of steps necessary and reduce the risk of contamination.
7. The inactivation step of proteinase K is important, as any residual proteinase K activity will digest the *Taq* polymerase, resulting in no or suboptimal amplification. The spinning step will ensure that all proteinase K solution will stay at the bottom of the Eppendorf tube.
8. Appropriate positive and negative controls are crucial. Apart from the usual reagent blank PCR control, known negative samples should be subjected to all the sample processing steps in order to control for possible contamination during these stages. Positive controls should include known specimens with the target of interest. In addition, each sample should be subjected to a control amplification, using a sequence which is present in all samples, e.g. the beta-globin gene, to test for the quality of the samples It should be noted that the control and test targets should be of similar lengths, so that they will be affected similarly by fixation parameters and the age of the specimen. Provided that the target is of similar copy number to the control, the two targets (test and control sequences) can be coamplified. In situations where the two are of significantly different copy numbers, e.g., certain viral targets, it is advisable to carry out the control and test amplifications separately
9. If there is no PCR signal, the following measures may be taken:
   a Adjust the amount of specimen extract used for PCR. Suboptimal amount of specimen extract will obviously give rise to less than ideal amplification.

However, too much specimen can also inhibit PCR due to the presence of inhibitors in tissue samples *(10,11)* . It is advisable, therefore, to use a range of sample volumes, e.g., from 1 to 20 µL per 100 µL PCR.

b. Increase the sensitivity of PCR: this can be achieved by increasing the number of cycles and/or by using the Hot Start technique *(12)*
c. Reassess the proteinase K inactivation steps: incomplete inactivation of proteinase K will result in the digestion of the *Taq* polymerase.
d. If the sample is still refractory to amplification, further DNA purification steps e.g., using phenol/chloroform extraction, will help in a significant proportion of cases *(11)*.
e. Even with the measures listed in (a) to (d), a proportion of archival specimens will remain refractory to amplification. The exact proportion will depend on the type of specimen, fixation method, and the age of the specimen.

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4

Quantitative PCR

Luc Raeymaekers

1. Introduction

Quantitative PCR recently has become a powerful tool in clinical investigations. Its main areas of application have been the assessment of residual disease after treatment of leukemia and lymphoma, the detection of viral nucleic acids, and the detection of gene amplification or deletion and of aneuploidy *(1)*.

The application of PCR as a quantitative tool requires the solution of the problem of how to reliably determine the initial amount of target template (T_0) from the amount of PCR product (T_n) that has accumulated after some number (n) of cycles. The relation between T_0 and T_n in most instances strongly depends on the PCR conditions. This difficult situation is caused by two main properties of PCR: first, the huge over-all amplification factor makes PCR very sensitive to small variations of the experimental conditions; the second problem is caused by the saturation phenomenon, i.e., the gradual decrease of the amplification efficiency that starts in the later stages of PCR, usually following the accumulation of some threshold amount of product.

In order to exploit the potential of PCR for high sensitivity, a typical PCR consists of many cycles. As a consequence, if there is a difference in the efficiency of amplification between separate PCR tubes or between different templates, the difference in the amount of product will be amplified as well. The error increases exponentially with n and with the magnitude of the difference between the amplification factors. For example, a DNA sequence amplified in one condition with an efficiency of 1 (i.e., the amplification factor in each cycle is 2, the maximum value theoretically possible) will generate, after 30 cycles, an amount of product that is 23.6-fold higher than in another condition in which the efficiency is 0.8 (i.e., the amplification factor in each cycle equals 1.8). After 40 cycles, the difference is a factor of 67.7.

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The other important variable is the effect of saturation of the PCR on the relation between T_0 and T_n. The amplification factor in each cycle remains constant up to some threshold value of T_n, which is reached after 15 to 30 cycles, depending on the amount of starting material. Thereafter, the amplification factor gradually decreases with n. Let us call this point the transition point, or *tr* point, and let us indicate the amount of product reached at *tr* as T_{tr}. The graph relating the logarithm of T_n to n is linear for values of T_n lower than T_{tr}. For values of T_n higher than T_{tr}, the curve gradually flattens until its slope is zero, corresponding to full saturation of the PCR. A consequence of this phenomenon is that if several reactions are run starting from different values of T_0, the initial differences will be preserved till T_{tr} is reached, but the difference will decrease from T_{tr} onward. Any difference in T_n will disappear completely if the PCR is run up to complete saturation. In other words, at a sufficiently large number of cycles, one will end up with always the same amount of product (we will indicate this amount by T_s), irrespective of the starting value T_0.

It is important to note that it has been observed that T_{tr} is sometimes much lower than T_s, up to a factor of 10^3 to 10^4 *(2)*. The amount of PCR product that is high enough to allow accurate quantification on ethidium bromide-stained gels, is, in these cases, greater than T_{tr} and should, therefore, not be used for absolute quantitative purposes without coamplification of a standard sequence. It can only be used for comparative purposes—for example to find out whether T_0 has increased or decreased. It is not sufficiently reliable to determine exactly by which factor this change has occurred. For all these reasons, much of the problem of quantitative PCR concerns the question of how adequate standardization can be achieved. The review of Ferre *(3)* is recommended reading for its extensive reflections on the factors that affect the quantitative power of PCR.

2. Materials

The only step in quantitative PCR requiring expensive equipment is the quantification of the PCR products. The widest dynamic range is obtained by direct imaging of gel bands using radioactively labeled deoxynucleotides (e.g., ^{32}P-αdCTP). There is a choice of apparatus on the market, in combination with the appropriate software, for quantification of the radioactive bands on polyacrylamide gels. The Packard InstantImager (Packard Instrument Company, Meriden, CT) measures the radiation directly, permitting readings within a few minutes. Others make use of the indirect method via exposure of the dried gel to a storage phosphor screen that is afterwards scanned by a laser, e.g., the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the Molecular Imager (Bio-Rad, Hercules, CA) apparatus (*see* **Note 1**). If such instruments

are not available, bands can be cut out from the gel and counted in a scintillation counter, although this is a tedious and less accurate procedure.

Fluorescence using classical fluorescent dyes such as ethidium bromide combined with densitometry is a cheaper and more convenient alternative—with a much more restricted dynamic range, however. Gel bands (preferably on polyacrylamide instead of agarose gels, *see* **Subheading 3.2.2.1.**) can be made fluorescent by using an intercalating dye (*see* **Note 2**) or by using a fluorescent primer for the PCR. The fluorescent bands are quantified by digitizing the image using a system equipped with a CCD video camera, such as the Eagle Eye apparatus (Stratagene, La Jolla, CA), and analysis with a suitable image analysis program, or by using a laser scanner designed for fluorescence imaging, such as the FluorImager from Molecular Dynamics. This apparatus, in combination with newly developed dyes, extends the dynamic range of the technique close to that of radioisotopes.

PCR in general, and quantitative PCR especially, require meticulous laboratory technique *(4)*. It is strongly recommended to use pipet tips with protective filters in order to avoid product carryover via the pipets (*see* **Note 3**). The PCR mixtures should be prepared in a room separate from that where amplified DNA is being handled and a separate set of pipets and pipet tips should be used. It is evident also that the accuracy of quantification depends on the accuracy of the volume of sample added. Therefore, small-volume, high-accuracy pipets should be available.

3. Methods

3.1. Quantification Without Coamplification of a Standard Sequence

This method of quantification requires the measurement in each PCR of the increase of T_n with n in order to check the duration of the exponential phase and to determine the amplification factor (A) during the exponential phase. The value of the logarithm of A is determined from the slope of the curve obtained by a linear regression analysis on the points relating the logarithm of T_n to n, according to the formula: $\log T_n = n \cdot \log A + \log T_0$, from which T_0 is calculated *(5)*. The advantage of this method is that there is no need to construct and test a standard sequence. The main disadvantage is that several samples have to be taken from the PCR mixture during the run. The method is therefore more suitable for one-time experiments than for the quantification of large series of samples. Since there are a large number of cycles between the sampling of the data points and the extrapolated value T_0, the accuracy of the result is strongly dependent on the statistical characteristics and accuracy of the regression analysis. It, therefore, requires very accurate quantification of the samples collected at the different time points. Care has to be taken not to include any data obtained beyond the *tr* point, as may occur in assays of samples in which T_0 is high.

3.2. Quantification Using a Coamplified Standard Sequence

Quantification by coamplification with a standard sequence is the most widely used method. This method allows to compensate for tube to tube variation in amplification efficiency. The amount of PCR product obtained from a specific template species (T_n) is compared to that amplified from a reference sequence or "standard" (S_n) in the same PCR tube. The standard is either an internal standard, i.e., a sequence that is constitutively present and the amount of which is considered to be invariant, or an exogenously added standard sequence (competitive PCR).

3.2.1. Internal Standards: Coamplification of the Target with a Control Gene

This method involves the coamplification of the target sequence with an unrelated sequence such as a single-copy gene or the transcripts of a housekeeping gene. The coamplification of unrelated sequences imposes strict constraints on the PCR conditions that allow reliable quantification, which may have not all been fulfilled in several published data. First, it requires the use of two primer pairs in one reaction. These four primers have to be checked for compatibility, i.e., for similar melting temperature and for absence of primer-primer binding. Second, the difference between the initial amount of target (T_0) and standard (S_0) should not be too excessive. The difference must be sufficiently small so that the amount of product of both templates will exceed the detection limit before the end of the exponential phase is reached, i.e., the difference between T_{tr} and S_{tr} should be within reasonable limits. Furthermore, the mRNA levels of the housekeeping genes may not remain constant in all conditions. Finally and foremost, both templates should amplify with the same efficiency, the most difficult requirement to be met.

In order to determine the copy number of amplified genes or the number of infecting virus particles, a sequence belonging to a single-copy gene is often coamplified as an internal standard. Two of the many examples from the literature are the analysis of the copy number of the dihydrofolate reductase (DHFR) gene in drug-resistant tumor cells ***(6)*** and of the *N-myc* gene amplification in neuroblastoma ***(7)***.

For the analysis of the relative quantity of gene expression at the mRNA level by RT-PCR, many authors have coamplified as an internal standard a fragment of a gene transcript that is considered to be invariant in the conditions considered. Genes that have often been coamplified are β-actin ***(8,9)***, glyceraldehyde phosphate dehydrogenase ***(10)***, and β_2-microglobulin ***(8)***. This method has been applied in a clinical context for example to the quantification of drug resistance-related gene expression in tumor cells ***(8)*** and for the assessment of viral activity ***(11)***.

3.2.2. Coamplification of the Target with an Exogenously Added Sequence: Competitive PCR

3.2.2.1. The Principle of the Method

The conditions for reliable quantification are much less stringent if the standard sequence is very similar to the target sequence. A minimum prerequisite is that the sequences for primer binding are identical in *T* and *S*. Equal amplification efficiency of *T* and *S* is further favored by similarity in length and in base composition. The method ***(12–14)*** has been called "competitive PCR" ***(14)*** because it allows, at least in principle but not always in practice, to extend the PCR beyond the exponential phase into the saturation phase, resulting in competition between both templates for amplification while preserving their initial ratio. The possibility to extend the PCR into the nonexponential phase is very useful in practice since it avoids the need of additional controls and allows the accumulation of sufficient PCR product for accurate quantification. It is the best method available. However, before embarking on the technique, it should be realized that the procedure is usually labor intensive and costly because many reactions have to be run and analyzed for one quantification.

The method of competitive PCR is shown schematically in **Fig. 1.** In theory, T_0 can be determined from the data obtained from only one PCR tube. If *T* and *S* are amplified with the same efficiency, the ratio of their products T_n/S_n will remain constant throughout the PCR and the value of T_n/S_n will remain identical to the initial ratio T_0/S_0. Since S_0 is added as a known quantity and T_n and S_n are measured, T_0 can be calculated from the equation $T_0 = (T_n \cdot S_0)/S_n$. In practice, however, the ratio T_n/S_n can be most accurately determined when its value does not differ greatly from 1. Therefore, as described by Gilliland et al. ***(14)***, the usual practice is to construct a standard curve from a dilution series of the standard. A PCR mixture is made containing all components (including the target sequence (T_0) to be quantified) except the standard template, and divided over several PCR tubes. To these tubes a 2- to 10-fold dilution series of the standard sequence (S_0) is added. Following amplification, both T_n and S_n are quantified. The standard curve is constructed by plotting the logarithm of the ratio T_n/S_n as a function of the logarithm of S_0 (**Fig. 1**). The value of S_0 is read at the point of equivalence, i.e., the point corresponding to $T_n/S_n = 1$ or $\log(T_n/S_n) = 0$. The quantity T_0 is equal to this value of S_0.

Since the sequences of *T* and *S* are very similar, the occurrence of the *tr* point will be determined by the sum $T_{tr} + S_{tr}$. Before the *tr* point is reached, the value of T_n in each PCR tube of the dilution series will be the same (assuming equal amplification efficiencies in all tubes), whereas the value of S_n will change in each adjacent tube according to the dilution factor of S_0. This situation is depicted in **Fig. 1**, gel bands labeled T_{tr} and S_{tr}. When the PCR is

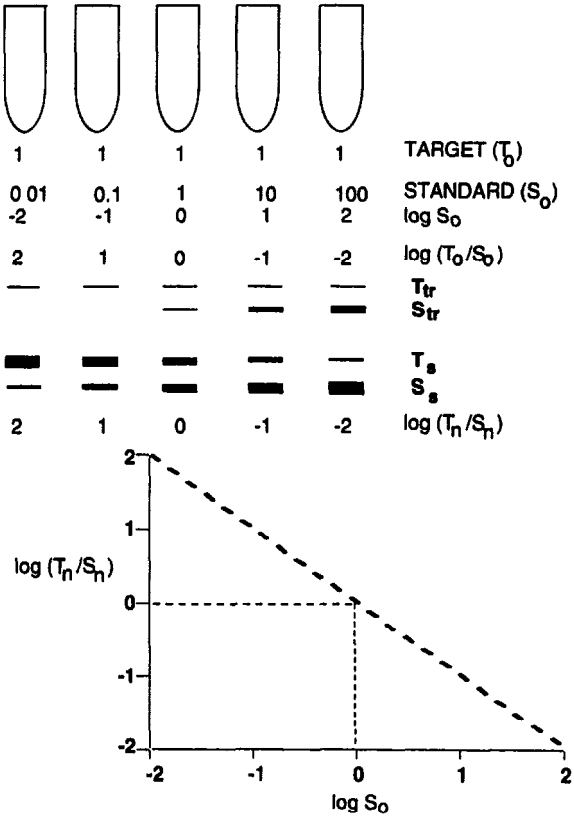

Fig. 1. Schematic representation of the method of competitive PCR. A series of PCR tubes containing an unknown amount of template (T_0), in the example tentatively set at 1, is spiked with a dilution series of a known amount of standard (S_0) (the respective values and the required logarithmic values are shown below each tube). The middle part of the figure schematically shows the gel bands obtained after amplification. T_{tr} and S_{tr} indicate the products obtained when the number of cycles is restricted to the purely exponential phase. T_{tr} is the same in each tube and S_{tr} differs in adjacent tubes by the same factor as does S_0. T_s and S_s represent the gel pattern obtained if the PCR is run up to saturation. In the latter case T_s and S_s change in opposite directions while still preserving their initial ratio. The graph at the bottom shows the standard curve constructed from $\log(T_n/S_n)$ and $\log S_0$. The value of T_0 is equal to the value of S_0 read at the point of equivalence.

run up to saturation, competition occurs between T and S. As a result, the values of T_n will not be equal in each PCR tube but will be smaller in tubes spiked with more S_0. A further consequence is that the values of S_n in each tube will differ by a factor which is smaller than the dilution factor (**Fig. 1**, bands labeled

T_s and S_s). This saturation phenomenon does not affect the standard curve because this curve is based on the ratio T_n/S_n in each tube, which in principle remains constant, whether or not competition occurs. For the same reason, the effects of tube to tube variation in amplification efficiency are eliminated.

As is clear from the example shown in **Fig. 1**, theory predicts that the standard curve of competitive PCR is a straight line with a slope of −1 (or a slope of +1 when plotting the inverse ratio S_n/T_n). This property has not been noticed by many authors. As a consequence, many published standard curves present a slope that is not equal to 1. There are two possible explanations for a deviating slope: First, there is a difference in the amplification efficiency of *T* and *S*. This difference is not of equal magnitude in all the PCR tubes of the dilution series (if the difference in amplification factor is the same in all the tubes, it can be easily seen from **Fig. 1** that the standard curve shifts in parallel without change of the slope). It has been suggested previously that such a situation may arise because the PCR tubes containing a higher quantity of S_0 sooner reach the *tr* point, and thus have spent a greater number of cycles in the saturation phase. Differences in amplification efficiency are more likely to occur in these later stages of the amplification process than in the beginning when the amount of product is small ***(15)***. Second, a systematic error in the quantification of faint bands induces a tilting of the standard curve without altering the position of the point of equivalence. In this case, the quantification is still correct if the point of equivalence, but no other point of the curve, is used for quantification. It has been observed that faint ethidium bromide-stained bands are underestimated when analyzed on agarose gels, resulting in a tilting of the standard curve ***(16)***. The analysis of the same samples on a polyacrylamide gel resulted in an orthodox standard curve of slope = 1.

It is clear, therefore, that polyacrylamide gels are better suited for the quantitative analysis of PCR products than agarose gels. Radioactive labeling is a better method than fluorescence for quantification over a broad range.

3.2.2.2 Construction of Standard Templates

The more the standard sequence resembles the target sequence, the greater the chances that both templates will amplify with the same efficiency in a variety of conditions, eventually including the saturation phase of PCR. A minimum requirement is that *T* and *S* possess the same sequences for primer binding. On the other hand, both sequences should differ in such a way that both products can easily be discriminated. The difference can be either a difference in length or the presence or absence of a specific restriction site (*see* **Note 4**).

The closest resemblance is evidently obtained by engineering a specific restriction site by mutation of one nucleotide. However, the presence of specific restriction sites in the target as well as in the standard, is preferable to

a specific restriction site in only one of both sequences because it allows to control for complete digestion and for heterodimer formation. This control is done by a double digest with both restriction enzymes (*see* **Note 5**).

A somewhat more divergent standard can be easily prepared if the sequence of interest is known in more than one animal species. In most cases it is possible to find conserved sequences suitable for primer binding sites and encompassing a stretch of nucleotides in which specific restriction sites are present. If the cDNA clone is not available, a fragment containing such a sequence can be amplified by PCR from material of the other species. Preferably a larger fragment is amplified by using primers located outside the region amplified in the competitive PCR.

The minimum resemblance is presented by standard sequences that have only the primer binding sites in common. The preparation of so called PCR MIMICS by ligation of primers and their complementary sequences to a restriction fragment of an unrelated sequence has been described by Siebert and Larrick *(17)*. PCR MIMICS for a whole range of interleukins and growth factors are available from Clontech (Palo Alto, CA). Constructs have also been made that contain a multitude of pairs of primer binding sites suitable for the amplification of a range of standards *(12,16,18)*. Thus one construct can be used as standard template for the quantification of a range of sequences.

When quantifying gene expression at the mRNA level by RT-PCR, an additional factor has to be taken into account. The reverse transcription step is a potential source of error because of variability in the efficiency of synthesis of the first strand cDNA. Therefore, several authors have added an exogenous cRNA standard to the RT reaction instead of a cDNA standard to the PCR mix. However, the use of RNA introduces other difficulties because of its susceptibility to degradation. Furthermore, one has to face the possibility that a RNA construct that is much shorter than the natural mRNA could be transcribed with a different efficiency because of, for example, a difference in secondary structure.

Whatever type of standard is used, the accuracy and reproducibility of the quantification is appreciably improved by always using the same batch of standard and by running all samples in the same PCR.

3.2.3. Other Strategies for Quantitative PCR

3.2 3.1. Limiting Dilution

Limiting dilution assays are used to determine the frequency of rare positive entities among a majority of negative entities. It has been applied in combination with PCR to quantify a particular DNA sequence relative to the number of

starting cells ***(19)***, or in combination with RT-PCR to estimate the fraction of cells expressing a particular gene ***(20,21)***. For the latter method to work it is necessary to isolate RNA after dilution of the cells ***(21)*** or to skip the mRNA isolation step and to perform the reverse transcription reaction directly on the diluted cells, e.g., as described by Molesh and Hall ***(20)***.

A dilution series is made of the sample in PCR tubes, extending up to a dilution in which the probability of finding a positive cell is very small. At each dilution, multiple (p) replicate samples are run. The results can be analyzed according to the single-hit Poisson model if the presence of one positive cell is sufficient to give a positive signal in a reproducible way. Therefore, many PCR cycles may be required, preferably divided over two steps in a nested PCR. In addition, great care is needed to avoid false positives.

Several methods have been described to calculate f, the estimate of the fraction of positive cells in the parent population. Since the description of these methods is beyond the scope of this chapter, only the simplest of these procedures are explained, although they are the least accurate ***(22,23)***.

The expected fraction of negative tubes at dilution i (F_i) is given by $F_i = \exp(-f \cdot c_i)$. This expression is the zero term of the Poisson equation, in which c_i = the number of cells per tube at dilution i. Since $f \cdot c_i = -\ln F_i$, the value of f corresponds to the slope of the regression line of a plot of $\ln F_i$ as a function of the number of cells per tube (c_i), fitted through the origin.

A first approximation of f can also be derived in a convenient way from the equation ***(20)***:

$$f = \Sigma k_i / [N - \Sigma(k_i \cdot c_i/2)] \quad (1)$$

in which N = the total number of cells in all the tubes at all dilutions ($N = p \cdot \Sigma c_i$).
k_i = the number of positive tubes at the ith dilution

To use this approximation, only the dilutions that produce mixed positive and negative tubes should be used along with, at most, one neighboring higher and lower dilution.

3.2.3.2. Ratio PCR

Ratio PCR is a simple method to analyze the relative expression level of mRNA transcribed from members of a gene family ***(24)***. Primers are selected in perfectly conserved regions in order to amplify all members expressed in the sample of interest. In order to allow the discrimination of each sequence in the PCR product, a further requirement is that the region encompassed by the primer binding sites contains a specific restriction site in each sequence. It proved possible to meet these conditions in all seven gene families examined up till now. Since this method is a kind of competitive PCR, all precautions required for the latter also apply to ratio PCR.

3.3. Remarks on the Measurement of the Total Amount of Starting Material

Besides the problem of the quantification by PCR of a specific target sequence in a complex sample, one has to deal with the additional problem of the measurement of the total amount of this starting material. This characterization implies as well the quantification of the amount of DNA or RNA, which may pose a problem especially in the case of small amounts of tissue as the quality control of the starting material. Quality control is particularly important for clinical samples since they are often obtained under less stringently controlled conditions than those prevailing in the research laboratory.

If the starting material consists of isolated cells, cell counting is a convenient way of quantification. For small amounts of tissue, the yield of DNA or RNA may be too low for spectrophotometric quantification. Low amounts of double-stranded DNA can be estimated by spotting and comparing the intensity of staining with ethidium bromide to that of a standard series *(25)*. Alternatively, the content of a single-copy gene or the expression of a housekeeping gene can be quantified by PCR.

4. Notes

1. It is not necessary to add the radioactive isotope at the start of PCR. It can be added at a later stage. PCR is then continued for a few but even number of cycles in order to reach an even distribution of the marker. The molar ratio of the individual bands is calculated from the sum of the content of the nucleotide that was spiked with the tracer and its complementary base, e.g., the CG content if ^{32}P-αdCTP has been added.
2. Evidently, to obtain molar ratios for products of different length, the measured values have to be corrected for the length-dependent incorporation of the intercalating dye.
3. Filter-containing pipet tips from some manufacturers cannot be filled to the normal maximal volume as their equivalents without filter. The use of such tips makes life unnecessarily difficult.
4. In addition, PCR products have been quantified by hybridization with specific probes. This method appears rather cumbersome and will not be farther considered here.
5. When digesting with both restriction enzymes in combination, the amount of PCR fragment remaining at the position in the gel corresponding to its original length should be negligible. Incomplete digestion by one of the restriction enzymes would result in erroneous results. Keep in mind that PCR mixtures are strongly buffered at a pH that is not optimal for most restriction enzymes The PCR sample should therefore be sufficiently diluted in the restriction buffer in order not to affect the pH too much. The dilution factor can be minimized by selecting restriction enzymes that are compatible with a high buffer concentration

Since the restriction enzymes are selected for selectively cutting one of the templates and not the other, the corresponding recognition sites will not be present in heterodimers. Thus, a double digest in the presence of heterodimers will be incomplete. Heterodimer formation is induced by high product concentration and, hence, by saturation of the PCR. In principle, the problem of heterodimer formation can be solved by diluting a sample in fresh PCR mixture and running one or a few more cycles

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5

Mutation Screening Using PCR-SSCP

Silver Staining and Isotopic Protocols

Philip J. Saker

1. Introduction

Screening for mutations prior to sequencing can reduce the time and costs of identifying mutations. When the DNA sequence is known, the technique of detecting mutations as single-stranded conformational polymorphisms (SSCP) is a convenient method of screening for possible mutations. SSCP was originally developed by Orita et al. *(1)*. It has the ability of detecting a single base change, and has been applied to a number of genes, including the insulin receptor *(2)*, GLUT 4 *(3)*, glucokinase *(4)*, and the mitochondrial genome *(5)*.

The principle of SSCP analysis is relatively simple. In nondenaturing conditions, single-stranded DNA (ssDNA) has a folded conformation that is determined by intramolecular interactions and therefore its base sequence. When electrophoresed on a nondenaturing polyacrylamide gel, the ssDNA will have a specific mobility depending on this base sequence. Any difference in the base sequence of an ssDNA sample, due to a mutation or polymorphism, will be detected as a mobility shift, and will produce a different band pattern when compared to the normal "wild-type" **(Fig. 1)**.

SSCP is a convenient and a relatively rapid method to identify those subjects likely to possess a mutation. Subsequent sequencing is required to determine whether a particular "abnormal" band pattern is due to a mutation or polymorphism. When optimized, SSCP is reported to have a sensitivity of between 85–95% *(1,6)*. The conformation of the ssDNA in the gel may be altered by a number of conditions that have to be optimized to detect the mutations. These variables include:

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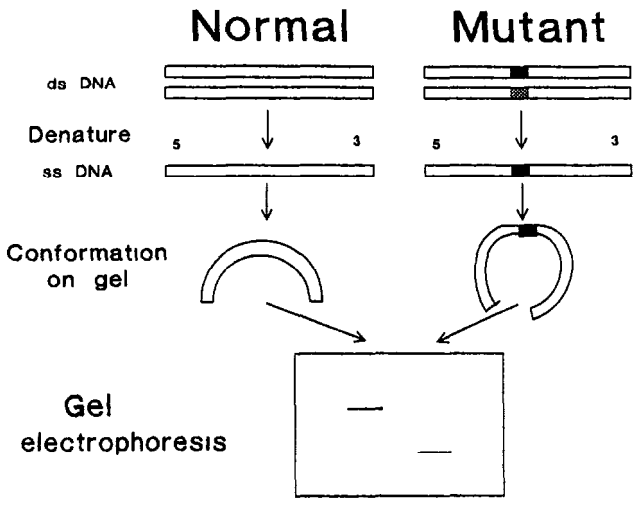

Fig. 1. Principles of SSCP. Heat denatured dsDNA to form ssDNA. The conformation of ssDNA is determined by its base sequence. A mutation or polymorphism will alter this, and affect its electrophoretic mobility. This will be seen as a difference in the SSCP band pattern compared to that of a normal ("wild-type") ssDNA sequence

1. Properties and pore-size of the gel; these are determined by the percentage of polyacrylamide, ratio of acrylamide/*bis* (cross-linker), and the temperature at which polymerization is carried out, including the temperature of the reagents.
2. Presence and percentage of glycerol; this has a weak denaturing action that partially opens the folded ssDNA structure so that more surface area is available for the gel to "sense" conformational changes
3. Temperature at which electrophoresis is performed.
4. pH of the buffer used to make the gel.
5. pH and ionic strength of the buffer system.

The SSCP band patterns may be detected by using radioactivity, silver-staining or fluorescence. This protocol will concentrate on silver-staining, because of its ease of use and safety within a laboratory not designated for using radioactive isotopes. Fluorescence-based SSCP will be covered in Chapter 6.

2. Materials

All solutions are made using deionized-distilled water. Purity of 16–18 *M*Ω per cm is recommended. Ultrapure or molecular biology grade chemicals and reagents are used.

2.1. Sample Amplification

1. 100 ng of DNA
2. 50 pmol of each primer.
3. 0.2 m*M* of each dNTP.

4 Buffer: 10 m*M* Tris HCl (pH 8 3 at 25°C), 50 m*M* KCl, 1 5 m*M* $MgCl_2$, 1% TritonX 100
5. 1 U of *Taq* polymerase.
6. For detection of SSCP bands using radioactivity Hot PCR is performed. Precautions need to be employed when handling radioisotopes, particularly the prevention of aerosols and avoidance of contamination. Local safety regulations need to be followed, along with the manufacturer's guidelines.
   The above reaction is used with the addition of 1 μCi of [α^{32}P] dCTP (3000 Ci/mmol, 10 mCi/mL) ***(4)*** or 0.2 m*M* of each dCTP, dGTP and dTTP, 2 nmol cold dATP; 0.4 μl [^{35}S] dATP (1000 Ci/nmol, 10 μCi/mL) ***(6)***.

2.2. Gel Preparation

1. 10X TBE: 1 liter, 108 g Trizma-base, 55 g boric acid, 9.3 g EDTA, pH 8.0. Store at room temperature (RT).
2 Ammonium persulfate: Store tightly sealed at room temperature (RT) for up to 1 yr. Use 25%$^{w}/_{v}$ solution, make fresh daily. Store solution at 4°C.
3. TEMED: *N,N,N',N'*-tetramethylethylenediamine. Store at 4°C. After 10–12 mo there is a significant reduction in activity
4. Glycerol: store at RT
5 Acrylamide/*bis* solution: 40% 19:1 acrylamide/*bis* solution Store at 4°C. WARNING. Acrylamide monomer is a neurotoxin (polyacrylamide is not toxic) absorbable through the skin; always wear gloves, avoid creating aerosols and dusts If in contact with skin, wash with soap and rinse thoroughly with water
6. Deionized-distilled water

2.3. Sample Preparation

1. Gel loading dye: 0.1% bromophenol blue, 0.1% xylene cyanol, 90% formamide and 20 m*M* EDTA in deionized-distilled water. Store at 4°C.
2. Deionized-distilled water

2.4. Silver Staining

1. Fixative 1: 40% methanol/10% acetic acid in deionized-distilled water. Store at RT (*see* **Note 20**).
2. Fixative 2: 10% ethanol/5% acetic acid in deionized-distilled water. Store at RT (*see* **Note 20**).
3. Oxidizer: 0.0032 *M* potassium dichromate and 0.0032 *M* nitric acid. Store at 4°C WARNING: Strong oxidiser, avoid contact with reducing agents; irritant, avoid contact with skin and eyes.
4. Silver reagent: 0 012 *M* silver nitrate. Store at 4°C. WARNING: Poisonous, caustic to eyes, skin and mucous membranes.
5 Developer: 0.28 *M* sodium carbonate and 0.5 mL of formalin per liter Store at 4°C, although it may be stored at RT for 1 month. WARNING: Poisonous. Irritant, vapor and dust irritates eyes, mucous membranes, and skin.
6. Whatman filter paper.

Table 1
Typical Volumes Required to Prepare a Polyacrylamide Gel

Electrophoresis temperature	4°C, mL	25°C, mL
10X TBE	3 mL	3 mL
Deionized-distilled water	19.5 mL	18.0 mL
Glycerol	—	1 5 mL
40% of 19:1 acrylamide/*bis* solution	7 5 mL	7.5 mL
TEMED	24 µL	24 µL
25% Ammonium persulfate solution	190 µL	190 µL

2.5. Autoradiography

1. Fixative 1: 10% methanol/5% acetic acid in deionized-distilled water.
2. Whatman filter paper.
3. Kodak X OMAT film
4. X-ray cassette, intensifying screens are not required.
5. –70°C Freezer.
6 Darkroom.

3. Methods

3.1. Sample Amplification

1. Amplify region of interest, usually the exons and approximately 30 bases flanking it, using the relevant primers and PCR. A fragment of 150–200 bp is optimal for SSCP (*see* **Note 1**) A typical PCR is carried out in a 50 µL volume using a thermal profile of 94°C 5 min; then 35 cycles of 94°C 30 s, 60°C 30 s, 72°C 30 s, and finally 72°C 10 min (*see* **Note 3**).
2. Confirm amplification on a 1.5% agarose gel with ethidium bromide staining, followed by illumination with UV light (*see* **Note 10**).

3.2. Preparation of Polyacrylamide Gels (see Note 18)

For SSCP, denatured samples are run on polyacrylamide gels at two different conditions. This is generally 10% polyacrylamide gel with 5% glycerol with electrophoresis at 25°C, and 10% polyacrylamide gel without glycerol with electrophoresis at 4°C.

1. Ensure deionized-distilled water is used for SSCP as chloride ions interfere with silver staining.
2. Allow acrylamide/*bis* solution and TEMED to warm to room temperature.
3. Make fresh 10X TBE and 25% ammonium persulphate solution (*see* **Note 14**).
4. Set up glass plates and spacers prior to casting gel
5. A 30-mL gel mix is sufficient for a 20 × 20 cm gel with 0.4-mm thick spacers (*see* **Note 6**). For a 10% polyacrylamide gel run at 4°C and 25°C, the volumes listed in **Table 1** are required.

6. The above reagents are put into a Pyrex beaker. Ensure that the glycerol is thoroughly mixed in solution prior to addition of acrylamide/*bis*, TEMED and ammonium persulphate Gently, but thoroughly, mix the gel solution.
7. The gels are poured at room temperature (*see* **Note 4**) and the toothed comb inserted into the top of the gel. The gels are left to polymerize for at least 2 h at RT prior to use (*see* **Note 5**).
8 After polymerization, carefully remove comb and flush out wells with 1X TBE before placing gel in electrophoresis equipment (*see* **Note 7**)

3.3. Sample Preparation and Electrophoresis

Gel loading dye containing formamide prevents the renaturation of the ssDNA after denaturation. Double-stranded DNA (dsDNA) is run with the samples to act as a marker and to distinguish its band from those created by ssDNA (*see* **Note 11**). If available, a positive control (PC) sample and normal "wild-type" sample are also loaded onto each gel (*see* **Notes 8** and **9**). The loading positions of the dsDNA and PC samples aid identification and orientation of the gel.

1. Samples are prepared as follows in labeled tubes:
   Sample tubes 16 µL dH_2O, 2 µL SSCP dye, 2 µL PCR product
   PC tubes. 16 µL dH_2O, 2 µL SSCP dye, 2 µL PCR product
2. The tubes are capped and centrifuged for a few seconds at 4°C
3. The samples are denatured at 95°C for 6 min. This may be done in a heated block, but it is more convenient to use a PCR thermal cycler
4. To prevent renaturation, the tubes are immediately placed on ice for 10 min.
5. Centrifuge the tubes again for 1 min at 4°C, and then immediately put back on ice
6. Prepare the dsDNA sample in a labeled tube: 17µL dH_2O, 2 µL SSCP dye, 1 µL PCR product.
7. The samples are loaded onto the polyacrylamide gel using a duck-billed pipet tip (*see* **Note 12**).
8. The gel is typically run at 25W for approx 18 hours in 1X TBE (*see* **Notes 13**, **15**, and **16**). A 200-bp ssDNA sample will run approximately three-quarters the way down the gel. The power may be altered to vary the run-time and separation (*see* **Note 17**).

3.4. Visualization of SSCP Bands

3.4.1. Silver Staining of Gels

SSCP bands may be visualized by silver staining (*see* **Note 27**). Clarity may depend on the amount of sample loaded onto the gel. The gel may have a dark background or be stained totally black if chloride ions are present in the water used to make the solutions. The following protocol is based on a method by Merril et al. *(7)*, and is commercially available in a modified kit (silver stain kit, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). The pro-

Table 2
Troubleshooting Guide for Silver Staining

Problem	Solution
Gray or brown precipitate appearing as smudges or swirling on gel surface. May become mirror-like. Bands may be faint or absent.	1. Nonspecific deposition of silver due to carryover of oxidizer or silver reagent Increase number and duration of wash steps 2. Temperature too low. Ensure all reagents are at least 23°C 3. Mirroring can be due to developer precipitate sticking to gel surface. First volume of development solution must be decanted as soon as precipitate appears
Dark uniform background, usually yellow.	Incomplete removal of oxidiser. Increase wash steps to remove all traces of yellow before addition of silver reagent.
Mottled background.	1. Contaminants in water. Check purity 2. Incomplete removal of gel buffer components, increase timing of Fixatives 1 and 2.
Slow or no development	1. Development rate is temperature-dependent. Developer solution may be heated to 50°C to speed up development. 2. Developer solution is too old 3. Ensure developer solution is mixed thoroughly.
Gel continues developing, or becomes darker when drying on Whatman filter paper.	Increase timing of **step 13** of **Subheading 3.4.1.** Repeat two to three times. As a safeguard, photograph gel prior to drying
Incomplete staining.	Complete silver staining, then recycle gel. Soak in deionized-distilled water for 30 min and then repeat **Steps 8–13** of **Subheading 3.4.1.**
Large discolored spots on gel	Pressure on the gel will cause the gel to stain darker at the contact point. Avoid crushing the gel with fingers etc.

cess is temperature-dependent, particularly the developer stage, and should be carried out at room temperature (*see* **Note 22** and **Table 2**).

The following steps have to be performed with care so as not to damage the gel. Gloves should be washed in deionized-distilled water to remove powder and other contaminants which may discolor the gel. If possible, lightly hold the gel at the top only; excessive handling and pressure will affect the staining.

1. Remove gel from glass plates and place face down in a 21 × 21 × 5 cm Pyrex baking dish.
2. Add 400 mL of Fixative 1; remove after 30 min (*see* **Notes 19, 21, 23–25** and **26**).
3. Add 400 mL of Fixative 2; remove after 15 min.
4. Repeat step 3.
5. Add 200 mL of oxidiser; remove after 5 min.
6. Add 400 mL of deionized water, gently agitate; remove after 5 minutes.
7. Repeat step 6 twice.
8. Add 200 mL of silver reagent; remove after 20 min.
9. Add 400 mL of deionized water; remove after 1 min.
10. Add 200 mL of developer, gently agitate; remove after approx 30 s when the solution turns yellow, or brown precipitate appears.
11. Add 200 mL of developer, may be gently agitated; remove after 5 min
12. Repeat step 11 if bands require further development.
13 Add 400 mL of Stop solution, leave for at least 5 min, then remove.
14. Gently place piece of Whatman filter paper, of sufficient size, onto the back of the moist gel. Starting from the top, carefully lift from baking dish. Gently place clingfilm onto front of gel and dry on a gel dryer
15. The gel may be stored or photographed.

Figure 2 shows a silver-stained SSCP gel.

3.4.2. Autoradiography

1. Remove gel from glass plates and place face-up in a 21 × 21 × 5 cm Pyrex baking dish.
2. Add 400 mL of Fixative 1; remove fixative after 30 min.
3. Gently place Whatman filter paper, of sufficient size, onto the front of the moist gel. Starting from the top, carefully lift from the baking dish. Gently place clingfilm onto the gel and dry on a gel dryer.
4. Place dried gel into an X-ray cassette and, in a dark room, load Kodak X OMAT film so that it is exposed to the gel surface. Leave at 70°C for 1–5 h.
5. Develop film (*see* **Note 28**).

4. Notes

1. Studies have shown that the optimum PCR length for SSCP is 150–200 bp, and that there is a reduction in sensitivity as the fragment increases ***(6)***. There may also be a minimum fragment length.
2. It is possible to amplify a longer PCR product and then cut this with a restriction enzyme into appropriate fragments. This is particularly useful when exons are separated by only a short length of intron, or if an exon is large. The two fragments can then be run together on the same SSCP gel. Twice the amount of digested PCR sample needs to be loaded.
3. If possible, optimize the PCR so that nonspecific primer binding does not occur as this could interfere with SSCP and cause confusion when interpreting the SSCP bands
4. Use of a 50-mL plastic syringe eases pouring of gels

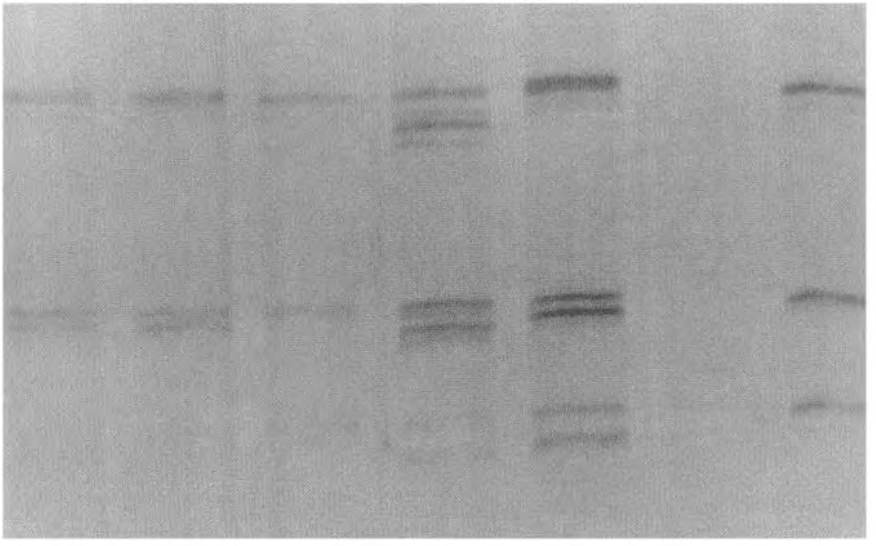

Fig. 2. SSCP gel using silver stain to detect bands. SSCP analysis of exon 8 of the glucokinase gene. Lane 1 is a negative control sample; lane 2 is blank; lane 3 is a positive control sample ($Gly^{299} \rightarrow Arg$) from a member of pedigree BX ***(12)***; lane 4 is a positive control sample from a French pedigree; lane 5 is a patient with a normal SSCP band pattern; lanes 6 and 7 are two patients with similar abnormal SSCP band pattens to the member of pedigree BX (lane 3). Sequencing confirmed that these two patients (lanes 6 and 7) possessed the $Gly^{299} \rightarrow Arg$ mutation ***(13)***.

5. Polymerized gels may be stored at 4°C prior to use; paper towels soaked in 1X TBE should be placed across the top of the combs, and then the gels wrapped in cling film. If run at 25°C, allow them to warm-up prior to loading.
6. Thinness or low percentage acrylamide may make the gels too weak to handle, which will be a particular problem when silver-staining.
7. Prior to loading of samples, the gels may be pre-run for approx 30 min.
8. Amplified DNA sample from a subject known to possess a mutation in the fragment being screened and a normal "wild-type" sample should be run to act as positive and negative controls, respectively. This will ensure that each gel is capable of resolving different conformers, and as indication that no problems have occurred with the gel, parameters or equipment during electrophoresis.
9. If appropriate positive controls are unavailable for the particular exon or gene being screened, it is preferable to run other samples of a similar length which possess a mutation, with the understanding that the conditions for resolving these conformers may not be optimal for the region being screened.

10. If the PCR product is of weak intensity on the agarose gel, it may be possible to load more product onto the SSCP gel rather than having to repeat the PCR.
11. The dsDNA may aid identification of the gel if more than one gel is run at once; load dsDNA in well 1 of gel 1, in well 2 of gel 2, and so on.
12. The same pipet tip may be used for loading each sample if it is thoroughly flushed out with deionized-distilled water between samples.
13. To maintain the temperature at which electrophoresis is performed, it is recommended one use a gel tank with a water-cooled chamber and a water-circulator with temperature control (e.g., LTD6G from Grant Instruments, Cambridge, Royston, Hertfordshire, UK).
14. Fresh 25% ammonium persulphate solution is recommended, but a stock may be made and store at 4°C if preferred
15. Varying the temperature at which electrophoresis is performed, the percentage of polyacrylamide and content of glycerol in the gel can affect the presence or absence of SSCP bands.
16 6% polyacrylamide gels are often used as an alternative to 10% gels, and may be run for only 6 h at 25W. The disadvantage is that they are more delicate and liable to tear during silver staining
17 In our experience, the optimized conditions may vary between laboratories and even technicians Other research groups have reported similar experiences ***(8)***
18. Some researchers have found that gels other than polyacrylamide, e.g., MDE gel (Hydrolink, FMC BioProducts, Rockland, ME) are better at resolving SSCP variants ***(9)***
19. After placing the gels in a glass baking dish, they may be stored for at least 2 d in Fixative 1 and the container sealed with cling film.
20. It is not essential to add 5% acetic acid in Fixative 1 or 2
21. Adjust duration of immersion for thicker or thinner gels accordingly.
22. Trouble-shooting (modified from the BioRad protocol handbook with permission) (*see* **Table 2**).
23. To pour off solutions from gel, one can gently place the thumb and index finger of both hands at the top corners of the gel to hold it in the dish and prevent its movement. Angle baking dish so that the solution pours out from the end nearest the bottom of the gel. The gel will loosely adhere to the bottom of the dish.
24. Gel may roll-up when immersed in solutions, this will affect the staining process. To unroll, place the thumb and index finger of both hands at the top and bottom of the gel. Slowly move hands out from the center, to the sides of the gel
25. To prevent roll-up of gels, slowly pour solutions down the sides of the Pyrex baking dish
26. Bubbles under gel will effect staining. Gently agitate gel to remove large bubbles from underneath gel.
27. Automated systems for electrophoresis and silver-staining have been developed and used for SSCP ***(10,11)***

28. If bands are faint, expose the film for a longer time. Alternatively, there may be a problem with the activity of the radioisotope used. Addition of a higher concentration of labeled nucleotide in the PCR may compensate for the reduction in activity.

Acknowledgments

I would like to thank Andrew Hattersley for his drawing shown in **Fig. 1**, and guidance during my own development of PCR-SSCP.

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6

Multiple Fluorescence-Based PCR-SSCP Analysis with Primer-, Post-, and Internal-Labeling

Hiroyuki Iwahana and Mitsuo Itakura

1. Introduction

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis is a simple and sensitive method to detect DNA alterations including even one point mutation. A disadvantage of PCR-SSCP, despite its sensitivity, is the necessity to use radioisotopes. To avoid radioisotopes, silver staining was introduced for band detection *(1)*. In the fluorescent amplification refractory mutation system (ARMS)-SSCP using two different fluorescence-labeled primers, bands were visualized by UV-transillumination *(2)*. Hayashi et al. *(3)* and Takahashi-Fujii et al. *(4)* developed F-SSCP using an automated DNA sequencer and a fluorescence-based image analyzer, respectively. In their systems, however, band detection was dependent on a single species of fluorescence. Ellison et al. *(5)* reported a method to detect multiple fluorescence using Applied Biosystems model 373A DNA sequencer (ABI373A) (Perkin Elmer, Applied Biosystems Division [PE-ABD], Foster City, CA), which can detect four different fluorescent colors in the same lane without controlling gel-temperature. We designed and attached a gel temperature-controlling system to ABI373A and developed a sensitive method of multiple fluorescence-based PCR-SSCP (MF-PCR-SSCP) analysis in which we introduced three different methods to fluorescently label PCR-amplified DNA fragments *(6–8)*.

The concepts of various SSCP methods are schematically shown in **Fig. 1A–C. Figure 1A** shows MF-PCR-SSCP analysis with primer-labeling, in which fluorescently labeled primers are used to label PCR-amplified DNA fragments. **Figure 1B** shows MF-PCR-SSCP analysis with postlabeling, in which DNA fragments are fluorescently labeled after PCR-amplification.

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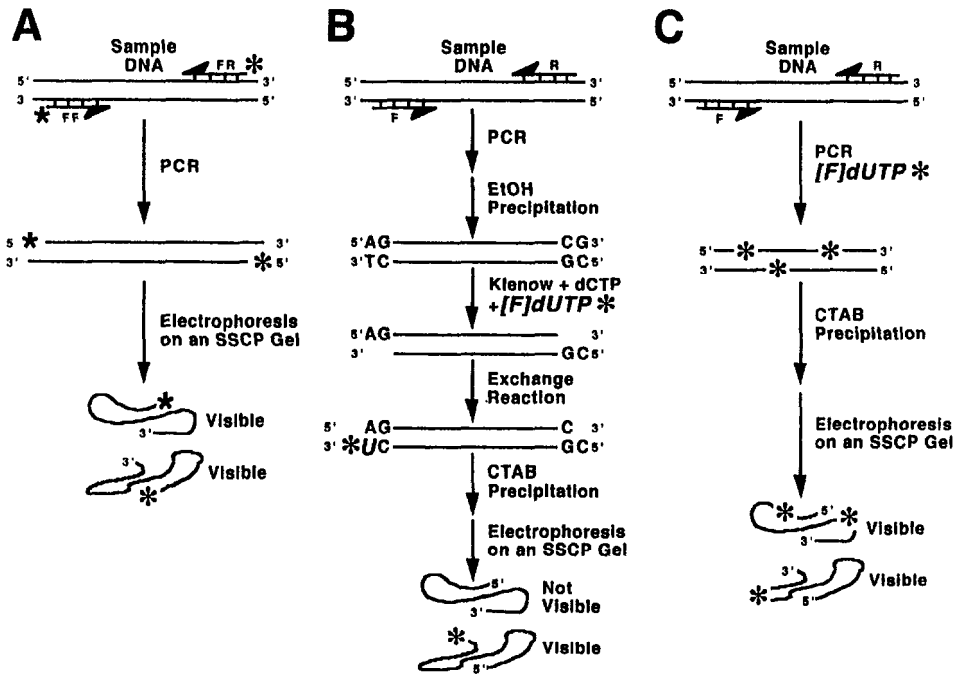

Fig. 1. Schematic presentation of the MF-PCR-SSCP methods. (**A**) Shows the flowchart of MF-PCR-SSCP analysis with primer-labeling. PCR-amplify the target sequence with a fluorescent forward (FF) and reverse primer (FR), each of which is labeled with different fluorescent dyes. The PCR product is applied to SSCP analysis using an automated DNA sequencer equipped with a gel-temperature controlling system and computer software, GENESCAN 672. (**B**) Shows the flowchart of MF-PCR-SSCP analysis with postlabeling. PCR-amplify the target sequence with a forward (F) and reverse primer (R). To label an antisense strand with [F]dUTP and to stop 3'→5' exonuclease activity of Klenow fragment at the 3'-penultimate position with dCTP, the 5'-ultimate and penultimate bases of a forward primer should be A and G, respectively. To prevent a sense strand from being labeled with [F]dUTP, G should be placed nearer to the 5'-end of a reverse primer than A. Remove unincorporated dNTPs by ethanol precipitation. The 3'-ultimate T of an antisense strand is exchanged with [F]dUTP using Klenow fragment. Unincorporated [F]dUTP is removed by CTAB precipitation. Then, perform SSCP analysis. (**C**) Shows the flowchart of MF-PCR-SSCP analysis with internal-labeling. PCR-amplify the target sequence with a forward (F) and reverse primer (R). To label the PCR product, add [F]dUTP to the PCR mixture. Unincorporated [F]dUTP is removed by CTAB precipitation. Then, perform SSCP analysis. The CTAB precipitation can be omitted in both post- and internal-labeling (*see* **Note 9**).

The nucleotides at the 3'-ends of PCR-amplified DNA fragments are replaced with fluorescently labeled deoxy- or dideoxy-ribonucleotides ([F]NTPs) by the exchange reaction using Klenow fragment of *Escherichia coli* DNA polymerase I. **Figure 1C** shows MF-PCR-SSCP analysis with internal-labeling, in which PCR-amplified DNA fragments are fluorescently labeled by adding fluorescent dUTPs ([F]dUTPs) into the PCR mixture.

MF-PCR-SSCP analysis with primer-labeling needs fluorescently labeled primers. However, it is simple and can detect sense and antisense strands separately. MF-PCR-SSCP analysis with postlabeling does not require fluorescently labeled primers and can detect sense and antisense strands separately. But its procedure is somewhat complicated. Although MF-PCR-SSCP analysis with internal-labeling also does not require fluorescently labeled primers and costs the least, it cannot differentiate sense and antisense strands and its resulting bands are broader than those with other two methods.

MF-PCR-SSCP analysis is a simple and efficient method to detect DNA sequence alterations. This method has several advantages including availability of internal DNA size markers, coelectrophoretic analysis of control and sample DNAs labeled with different colors, direct entry of the image data into the computer, and nonisotopic labeling. Fluorescent nucleotides do not decay as isotopes do and MF-PCR-SSCP analysis does not need X-ray films. Because of these advantages, MF-PCR-SSCP analysis is expected to enable the routine analysis of a large number of DNA samples as a part of genetic diagnosis.

2. Materials

1. Nonfluorescent primers and fluorescently labeled primers synthesized with an Applied Biosystems 392 DNA/RNA synthesizer (PE-ABD). Because of the detection limit of the length of DNA fragments in SSCP analysis, primers should be designed to amplify DNA fragments of shorter than 300-bp.
2. DNA samples to be examined.
3. Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP).
4. [F]NTPs (e.g., R6G-dUTP, R110-dUTP, TAMRA-dUTP, R6G-ddATP, R110-ddGTP, [PE-ABD]).
5. Reagents for PCR and a PCR thermocycler.
6. Reagents and an apparatus for agarose gel electrophoresis.
7. Reagents for ethanol precipitation (99.5% ethanol, 70% ethanol, and 5 *M* ammonium acetate).
8. Klenow fragment of *E coli* DNA polymerase I and H buffer (50 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), and 100 m*M* NaCl in a total volume of 20 µL) (*see* **Note 8**).
9. Reagents for cetyltrimethylammonium bromide (CTAB) precipitation: 5% CTAB; 1.2 *M* NaCl; 99.5% ethanol; and 70% ethanol.

10. Reagents for nondenaturing polyacrylamide gel (SSCP gel): 40% acrylamide (acrylamide: *N',N'*-methylene*bis*acrylamide- 50:1), 10X TBE buffer (1X TBE buffer is composed of 89 m*M* Tris base, 89 m*M* boric acid, 2 m*M* EDTA); 3% ammonium persulfate; and *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 50% glycerol when needed.
11 Internal DNA size markers e.g., GENESCAN-2500 ROX, GENESCAN-2500 FAM, GENESCAN-1000 ROX, GENESCAN-350 ROX, etc (PE-ABD) (*see* **Note 4**).
12. Loading buffer: Formamide and 50 m*M* EDTA, pH 8 0, are mixed at the ratio of 5.1. Blue dextran is added to 50 m*M* EDTA at the concentration of 0.03 g/mL to facilitate the application of DNA samples on the SSCP gel
13. A computer software of GENESCAN™ 672 (PE-ABD).
14 A system to control gel temperature of ABI373A (*see* **Note 5**) We developed two different systems in cooperation with Astec (Fukuoka, Japan) and Perkin Elmer Japan, Applied Biosystems Division (PEJ-ABD, Osaka and Fukuoka, Japan) ***(6,7)***. One system consisted of an aluminum block and a temperature-controlling unit, COOLNIT CL-150 (Taitec, Saitama, Japan). The aluminum block was 168.7 × 260 × 20 mm and drilled with ø12 mm holes. The temperature of an aluminum block, which was in direct contact with the back of the gel plate, was controlled by the circulating temperature-controlled ethylene glycol solution by COOLNIT CL-150 The gel-temperature was strictly controlled between –10 and 70°C with the accuracy of ±0 2°C. The other system consisted of a regulator unit and a one-touch attachment unit The surface of the attachment unit was temperature-controlled with Peltier elements and placed onto the surface of the front glass plate of the gel. The gel temperature was controlled between 10 and 60°C with the accuracy of ±0 5°C

3. Methods

3.1. MF-PCR-SSCP with Primer-Labeling

3.1.1. SSCP Gel

1. Wash the gel plates thoroughly with a detergent that contains no fluorescence Wipe the plates with soft paper. Rinse them with 99.5% ethanol and wipe them carefully with soft paper, such as Kaydry Wipers 132-S (Kimberly-Clark, Tokyo, Japan). Align the plates and spacers, and clamp at both sides to hold plates in position, while placing them horizontally on a flat surface (*see* **Note 1**).
2 To make 40 mL of X% SSCP gel mixture, mix X mL of 40% acrylamide, 4 mL of 10X TBE buffer, 840 μL of 3% ammonium persulfate, and 24 μL of TEMED (*see* **Note 2**). When necessary, 50% glycerol is added at the indicated concentration. Before adding TEMED, filter SSCP gel mixture through 0.45 μ*M* cellulose nitrate filter in a cup filter unit under vacuum, and then degas it for five minutes. Add TEMED and gently swirl the mixture.
3. Using a 10-mL syringe, pour the solution immediately and carefully between the plates. Insert a spacer at the top of the plates to make wells. Tightly clamp the glasses on spacers and cover the top and bottom with Saran Wrap. Wait for 2–3 h to ensure complete polymerization and set the gel plates to an automated DNA sequencer of ABI373A. Replace the top spacer with a shark comb.

3.1 2. PCR Amplification

1 PCR-amplify the target sequence with a pair of fluorescently labeled primers and 0 1 μg of sample genomic DNA as a template in a total volume of 10 μL The concentration of each primer is 0 1 μ*M* (*see* **Note 3**)
2 Cycle profiles of PCR-amplification are as follows. The primers to examine the K-*ras* gene are shown in the legend of **Fig. 2A** 30 main cycles 94°C, 0 5–1 min (denaturation); 55°C, 0 5–1 min (annealing), and 72°C, 1–2 min (extension), final extension· 72°C, 10 min

3.1.3 SSCP Analysis

1 Add 90 μL of loading buffer to fluorescently labeled PCR-amplified DNA fragments in 10 μL Heat the mixtures for 3 min at 90°C and immediately cool them on ice
2 Apply 1–5 μL of the solution to an SSCP gel fitted to the automated DNA sequencer that is equipped with a gel temperature control system Coelectrophorese the mixture of an internal DNA size marker such as GENESCAN-2500 ROX and DNA samples in the same lane (*see* **Note 4**)
3 Perform the data collection and analysis with a computer program of GENESCAN 672 (*see* **Note 6**) The results are shown as a gel image or an electropherogram As shown in **Fig. 2**, a gel image and an electropherogram show the migrated positions of DNA fragments as colored bands and peaks, respectively

3.2. MF-PCR-SSCP with Postlabeling

3.2 1 PCR Amplification

1 PCR-amplify the target sequence using 0 1 μg of sample genomic DNA as a template in a total volume of 50 μL The base at the 5'-ultimate position of primers is designed to be complementary to an [F]NTP scheduled to be used in postlabeling To stop 3'→5' exonuclease activity of Klenow fragment at the 3'-penultimate base with a nonlabeled nucleotide, the 5'-penultimate base of each primer should be different from the 5'-ultimate base The concentration of each primer is 1 μ*M* in all PCRs
2. PCR-amplify the target sequence under the same condition described in **Subheading 3.1.2.** The primers to examine the K-*ras* gene are shown in the legend of **Fig. 2B.**
3 Electrophorese 10 μL of the PCR product on a 1% agarose gel and confirm the amplification of the target DNA fragments

3.2 2 Postlabeling with [F]NTPs

1 Ethanol-precipitate the remaining 40 μL of the PCR-amplified DNA fragments In brief, add 40 μL of 5 *M* ammonium acetate and 200 μL of 99 5% ethanol. Mix by vortexing and place them at –20°C at least for 30 min Centrifuge at 18,500*g* at 4°C for 20 min. Discard the supernatant, wash the pellet with 70% ethanol, and dry the pellet
2. Dissolve the precipitated PCR-amplified DNA fragments in 30 μL of deionized and autoclaved water (DA-water) Determine the amount of DNA using a spectrophotometer

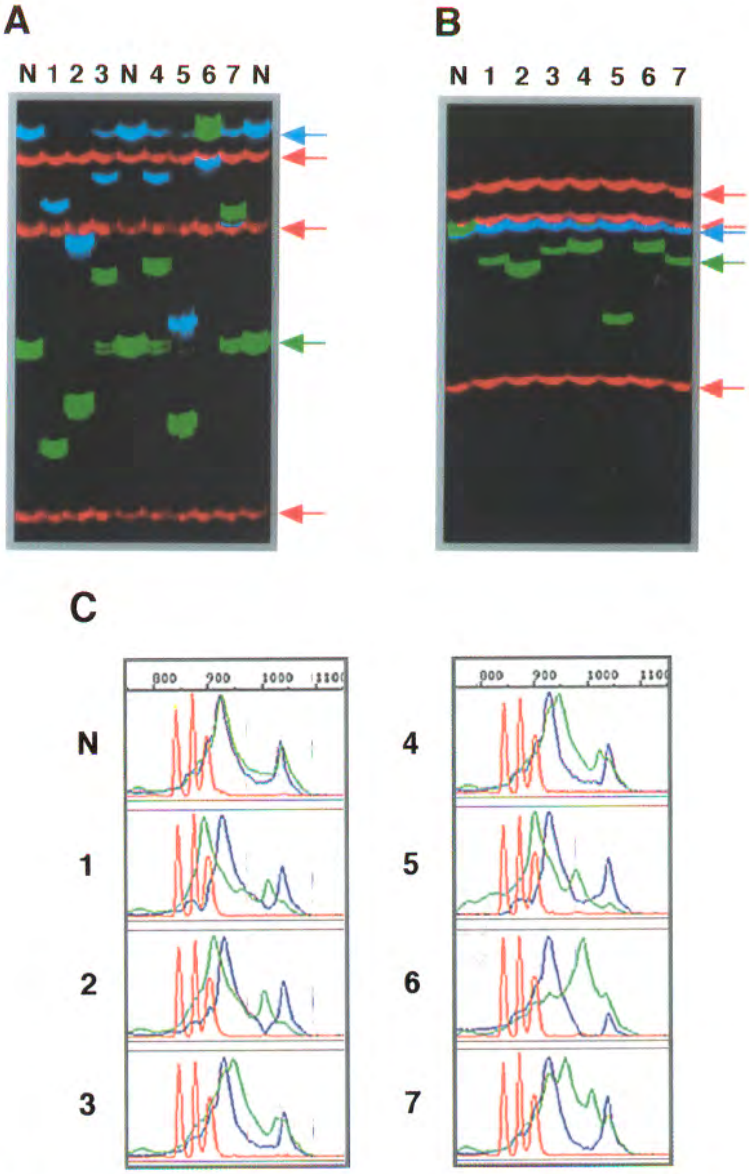

Fig. 2. Representative results of MF-PCR-SSCP with primer-, post-, and internal labeling. Six human tumor cell lines, A549, Lu65, PANC1, PSN1, SW480, and SW1116, which have nucleotide substitutions from GGT to AGT, TGT, GAT, CGT, GTT, and GCT, respectively, at codon 12 of the K-*ras* gene, and a human tumor cell line of MDA-MB231, which has a nucleotide substitution from GGC to GAC at codon 13 of the K-*ras* gene, were examined. Peripheral white blood cells obtained from a healthy subject were used as a normal control. GENESCAN-2500 ROX was used as

3 Postlabel the 3'-end of the PCR-amplified DNA fragment with Klenow fragment in the H buffer containing 50 m*M* Tris-HCl, pH 7 5, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), and 100 m*M* NaCl in a total volume of 20 μL (*see* **Note 8**)

Contents· 0 1-μg PCR-amplified DNA fragment; 0 05–0.5 μ*M* R6G-dUTP, 125 μ*M* dCTP, 2 U Klenow fragment, Incubation 30°C, 30 min

The base sequence at the 5'-ultimate and penultimate positions of A and G, respectively (5'-AG—3'), is taken as an example (**Fig. 1B**) In this case, either R110-dUTP (0 05–0 5 μ*M*) or TAMRA-dUTP (0 2–2 μ*M*) can be used for postlabeling If the 5'-ultimate base is T or C, [F]NTPs must be R6G-ddATP

Fig 2 *(continued)* an internal DNA size marker (**A**) Shows the result of MF-PCR-SSCP with primer-labeling in a gel image The 208-bp DNA fragment containing K-*ras* codons 12 and 13 was amplified using a FAM (blue)-labeled forward primer, FAM-TAATA-CGACTCACTATAGGGAGAGGCCTGCTGAAAATGACTGA-3', and a JOE (green)-labeled reverse primer, JOE-AATTAGGTGACACTATAGA-ATAGGTCCTGCACCAGTAATATGC-3' The underlined sequences indicate T7 and SP6 phage promoter sequences, respectively SSCP analysis was performed using a 10% SSCP gel of 24 cm at 20°C Blue, green, and red arrows indicate the migrated positions of the sense strand of normal control, the antisense strand of the normal control, and an internal DNA size marker of GENESCAN-2500 ROX, respectively (**B**) Shows the result of MF-PCR-SSCP with postlabeling in a gel image The 175-bp DNA fragment containing K-*ras* codons 12 and 13 was amplified by the nested PCR using pairs of primers of oRB492 (5'-ACCTTATGTGTGACATGTTC-3') and oRB493(5'-TGAAAATGGTCAGAGAAACC-3'), and oRB1050(5'-TGA GGCCTGCTGAAAATGACTG-3') and oRB1078 (5'-AGAAAGAATGGTC CTGCACCAG-3') The sense strand of normal control was labeled with 0 5 μ*M* of blue R110-dUTP and the sense strands of normal control and 7 tumor cell lines were labeled with 0.5 μ*M* of green R6G-dUTP The R110-dUTP-labeled normal control and the R6G-dUTP-labeled DNA fragments of normal control and tumor cells were mixed and applied to SSCP analysis using a 12% SSCP gel of 6 cm at 20°C Blue, green, and red arrows indicate the migrated positions of the sense strand of normal control, the sense strand of sample DNA of SW1116 as an example abnormal band, and an internal DNA size marker of GENESCAN-2500 ROX, respectively (**C**) Shows the result of MF-PCR-SSCP with internal-labeling in an electropherogram The 121-bp DNA fragment containing K-*ras* codons 12 and 13 was amplified by the nested PCR using pairs of primers of oRB492-oRB493 and oRB1050-oRB1633 (5'-AGGTTGGATCATATTCGTCCAC-3'). The DNA fragment amplified from a normal control subject was internally labeled with blue R110-dUTP and those amplified from a normal control and seven tumor cell lines were internally labeled with green R6G-dUTP. The control DNA fragments and sample DNA fragments were mixed at the ratio of 2 1 and coelectrophoresed on a 13% SSCP gel of 6 cm at 20°C. Blue, green, and red peaks indicate the migrated positions of control DNAs, sample DNAs, and an internal DNA size marker of GENESCAN-2500 ROX, respectively Lane N shows normal control. Lanes 1–7 show A549, Lu65, MDA-MB231, PANC1, PSN1, SW480, and SW1116, respectively.

(0 075–1.5 μM) or R110-ddGTP (0.02–0 4 μM), respectively. The 5'-penultimate base must be different from the 5'-ultimate base If the 5'-penultimate base is A, C, or T, the complementary nonlabeled nucleotide must be dTTP, dGTP, or dATP, respectively

4 CTAB-precipitate fluorescently-labeled DNA fragments. In brief, add 2.5 μL of 5% CTAB to the 20 μL solution and mix them by vortexing Centrifuge at 16,000*g* at room temperature for 3 min Discard the supernatant. Dissolve the pellet with 50 μL of 1 2 *M* NaCl and add 125 μL of 99 5% ethanol. Mix it by vortexing Centrifuge at 16,000*g* at room temperature for 3 min Discard the supernatant Wash the pellet with 500 μL of 70% ethanol and centrifuge under the same condition Dry up the pellet and dissolve it with 5–20 μL of loading buffer

3.2 3. SSCP Analysis

Make an SSCP gel as described in **Subheading 3.1.1.** Perform SSCP analysis as described in **Subheading 3.1.3.**

3 2.4. SSCP Analysis with Coelectrophoresis

Postlabel control DNA and sample DNA with different fluorescent dyes such as R6G-dUTP and R110-dUTP Mix them and coelectrophorese the mixture in the same lane. Perform data collection and analysis as other MF-PCR-SSCP analyses

3.3. MF-PCR-SSCP with Internal Labeling

3.3.1. PCR Amplification

1 PCR-amplify the target sequence with a pair of nonlabeled primers using 0.1 μg of sample genomic DNA as a template in a total volume of 10 μL The concentration of each primer is 1 μ*M* in all PCRs To internally label DNA fragments with fluorescent dyes during PCR-amplification, add 0 05–0 2 μL of [F]dUTP (R6G-dUTP, R110-dUTP, and TAMRA-dUTP at the final concentration of 0 5–2, 0 5–2, and 2–8 μ*M*, respectively) to the PCR mixture

2. PCR-amplify the target sequence under the same condition described in **Subheading 3.1.2.** The primers to examine the K-*ras* gene are shown in the legend of **Fig. 2C.**
3. Perform CTAB precipitation to remove unincorporated [F]dUTPs as described in **Subheading 3.2.2., step 4.**

3.3.2. SSCP Analysis

Make an SSCP gel as described in **Subheading 3.1.1.** Perform SSCP analysis as described in **Subheading 3.1.3.**

3.3.3. SSCP Analysis with Coelectrophoresis

Internal-label control DNA and sample DNA with different [F]dUTPs such as R6G-dUTP and R110-dUTP. Mix them and apply them to SSCP analysis.

4. Notes

1. Because the assembled glass plates are kept horizontally, it is not necessary to seal the bottom of the plates. When you pour the SSCP gel mixture between the plates or insert a spacer at the top of the plates, be careful not to make air bubbles When you replace the top spacer with a shark comb, wash the well carefully not to leave gel fragments, and insert a shark comb not too shallow or too deep
2. The resolution of SSCP analysis is improved with the increase of the polyacrylamide concentration and length of the gel. However, it takes a longer time with the thicker and longer gel. We usually use a 12–13% SSCP gel of 6 cm in length.
3. To save primers, reduce the amount of fluorescently labeled primers to one tenth of the amount used in the usual PCR.
4. GENESCAN-2500 ROX is a double strand DNA size marker. When it is mixed with loading buffer and heat-denatured, it separates into single strands and shows many bands on an SSCP gel. To prevent GENESCAN-2500 ROX from separating into single strands, mix 0 5 µL of GENESCAN-2500 ROX and 0 5 µL of 2X agarose gel loading buffer, which is included in a package of GENESCAN-2500 ROX, and apply it on an SSCP gel 1–5 min earlier than the heat denatured sample DNA. Because GENESCAN-350 ROX is a single strand labeled DNA size marker, it can be mixed with sample DNAs, heat-denatured, and electrophoresed on an SSCP gel.
5 Gel temperature is an important resolution factor in SSCP analysis. At 10°C, moisture might appear on the glass plates and disturb data collection. To determine the best temperature, check temperature between 10 and 30°C. In our experience, 20°C was the best to detect 7 different K-*ras* mutations.
6. Because a computer software such as GENESCAN 672 requires data of at least 400 scan numbers for analysis, it takes more than 40 min for data collection. To adjust the migrated positions with the internal DNA size marker, at least three peaks of an internal DNA size marker must be included.
7. The nested PCR is recommended both to obtain the single target PCR product and to enable the repeated examination with the small amount of a starting DNA sample.
8. The H buffer is the high buffer for restriction endonucleases. In our experience, the H buffer is better for postlabeling than the Klenow buffer which consists of 10 m*M* Tris-HCl, pH 7 5, 7 m*M* $MgCl_2$, and 1 m*M* DTT
9. The CTAB precipitation can be omitted in both post- and internal-labeling In this case, a broad nonspecific free [F]NTP band appears around the positions of standard DNAs of 850–1200-bp
10 Because the components of DNA sample solution such as salt concentrations affect SSCP profiles, coelectrophoresis, in which control and sample DNAs are mixed, is important and useful for accurate analysis. The different species of fluorescent dyes exert little effect on SSCP profiles.

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7

The Amplification Refractory Mutation System

Y. M. Dennis Lo

1. Introduction

The amplification refractory mutation system (ARMS) is an amplification strategy in which a polymerase chain reaction (PCR) primer is designed in such a way that it is able to discriminate among templates that differ by a single nucleotide residue *(1,2)*. ARMS has also been termed allele-specific PCR *(3)* or PCR amplification of specific alleles (PASA) *(2)*. Thus, an ARMS primer can be designed to amplify a specific member of a multi-allelic system while remaining refractory to amplification of another allele that may differ by as little as a single base from the former. The main advantage of ARMS is that the amplification step and the diagnostic steps are combined, in that the presence of an amplified product indicates the presence of a particular allele and vice versa. For routine diagnosis, this characteristic of ARMS means that it is a very time-efficient method. However, this combination of the amplification and diagnostic steps has resulted in a system that may not be as robust as some of the other methods in which these two important steps are separated, e.g., PCR followed by restriction enzyme analysis.

ARMS is based on the principle that the *Thermus aquaticus* (*Taq*) polymerase, the DNA polymerase commonly used in PCR, lacks a 3' to 5' exonuclease activity and thus a mismatch between the 3' end of the PCR primers and the template will result in greatly reduced amplification efficiency (**Fig. 1**). Thus, an ARMS typing system can be designed by constructing primers with their 3' nucleotide overlying the polymorphic residue. Hence, one ARMS primer can be constructed to specifically amplify one allele of a multiallelic system. For typing a system with *n* alleles, *n* ARMS primers will be required with the typing achieved in *n* reactions. For many diagnostic applications, a second set of control primers are also included in the PCR, and act as an internal amplification control.

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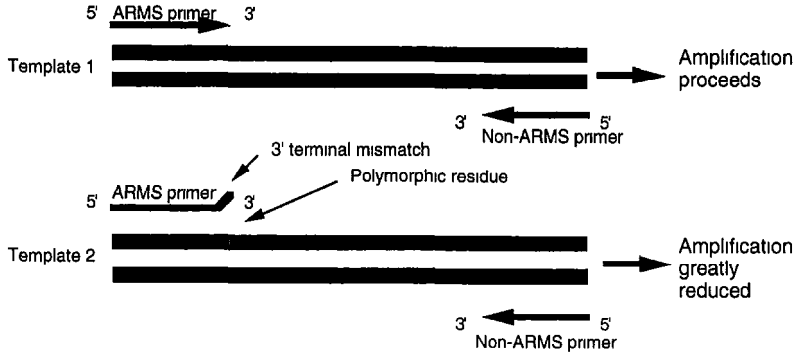

Fig 1

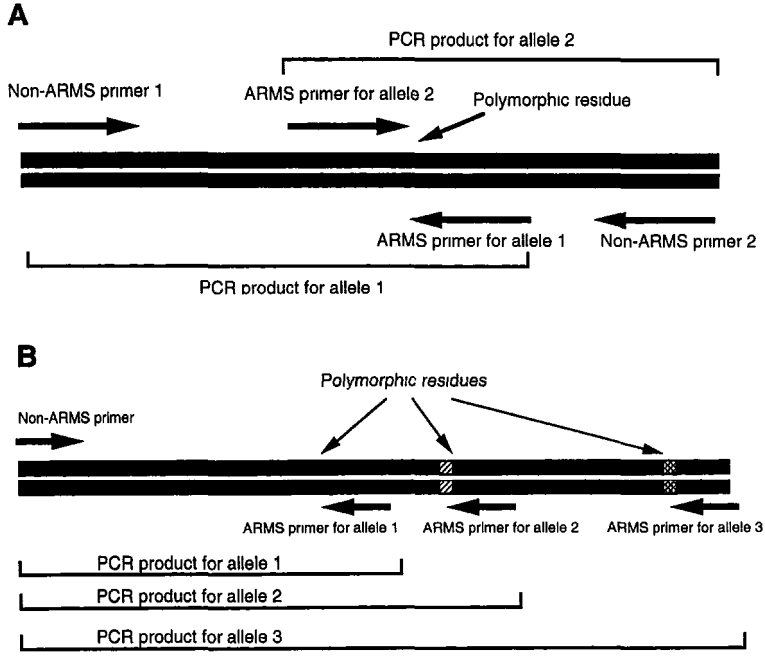

Fig. 2

Variants of the ARMS strategy have been described: multiplex ARMS *(5–7)* and double ARMS *(8)*. Multiplex ARMS is developed to facilitate the application of ARMS to genotyping multiallelic systems. In multiplex ARMS, a number of ARMS primers, each specific for a particular allele, are included in a single reaction in a single tube. PCR products corresponding to different alleles can then be distinguished by physical characteristics, such as length. A number of formats for multiplex ARMS are potentially possible. Two of these configurations are illustrated in **Fig. 2.**

In double ARMS, two allele-specific ARMS primers are simultaneously used in a single reaction (**Fig. 3**) ***(8)***. Amplification will only occur when the alleles specific for each of the ARMS primers are present on the same chromosome. Double ARMS is thus a very powerful system for elucidating the relationship of polymorphic sites, in other words, for clarifying the haplotype. This is very useful in determining the haplotype of individuals who are doubly heterozygous at two polymorphic sites (**Fig. 4**). This relatively simple approach is more efficient in many instances than the other methods for haplotype analysis, such as pedigree analysis and single molecule dilution ***(9)***, or single sperm typing ***(10)***. It is also very useful for genotyping multiallelic systems in which individual alleles are distinguished from each other by having different combinations of polymorphic residues as a positive signal is only present when the right combination of ARMS primers is used. In this respect, double ARMS is very useful for human leukocyte antigen (HLA) typing ***(5)***. Double ARMS also has a greatly enhanced specificity compared with single ARMS. This characteristic is important when the ARMS-based approach is used for the detection of a minority DNA population among a background of related but nonidentical DNA molecules ***(11)***. In these scenarios, the relative, rather than absolute specificity of conventional single ARMS may not be enough for the detection of minority DNA population that is present at a level of 1/1000 or below compared with that of the background DNA population. In these situations, the low concentration of the target DNA would require a high cycle number that would provide opportunity for the inefficient mismatched ARMS reaction to take place. Once the ARMS reaction has taken place, the mismatched reaction will proceed exponentially because the ARMS primer will become incorporated into the final PCR product, thus giving rise to a false-positive result. For a double ARMS-based system, however, mispriming by both ARMS primers (a highly unlikely phenomenon) would be necessary before a false-positive reaction is

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Fig. 1. *(previous page, top)* Principle of single ARMS. The ARMS primer is designed such that it matches with the target allele (template 1) at the 3' end but is mismatched at the 3' end with the nontarget allele (template 2). A 3' terminal mismatch will greatly reduce the amplification efficiency A downstream non-ARMS primer is also included to allow PCR to proceed Reprinted with permission from **ref. *4***

Fig. 2. *(previous page, bottom)* Examples of multiplex ARMS formats. Multiplex ARMS is designed to reduce the number of PCR necessary to resolve multiallelic systems using ARMS. (**A**) A system developed for typing a biallelic system. Apart from the ARMS products for allele 1 and allele 2, there is also an internal positive control product formed by the two non-ARMS primers. To facilitate the differentiation of the ARMS products from the two alleles, the PCR products should be designed to be of different lengths. (**B**) A system developed for a triallelic system. The three PCR products are designed to be of different lengths. **Figure 5** demonstrates an assay using this concept. Reprinted with permission from **ref. *4***.

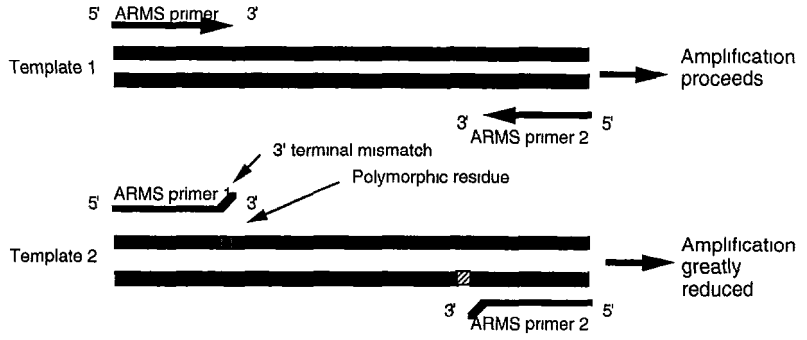

Fig 3. Principle of double ARMS Two allele-specific ARMS primers are included in a single PCR. ARMS primer 1 is designed to discriminate on the basis of polymorphic residue 1 and ARMS primer 2 is designed to discriminate on the basis of polymorphic residue 2. If either ARMS primer has a terminal mismatch with a template, then amplification efficiency is greatly reduced. Reprinted with permission from **ref. *4***.

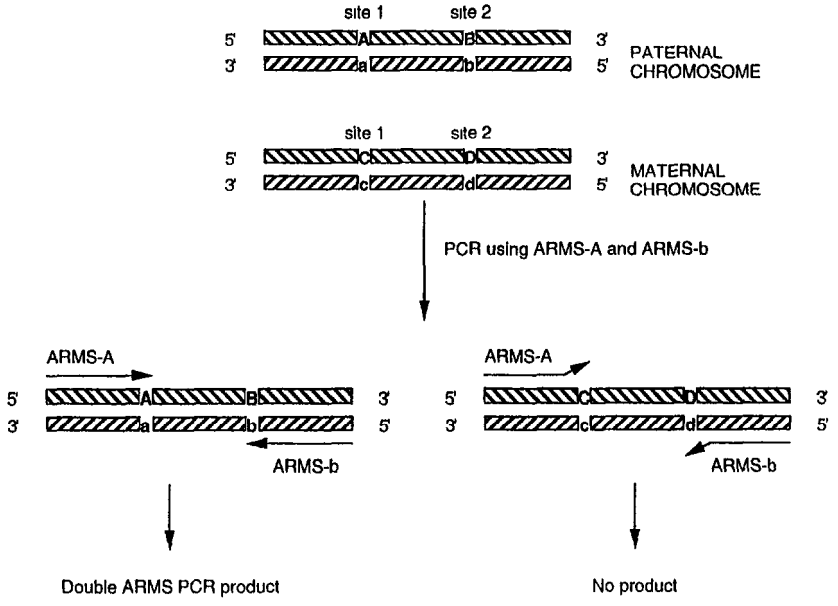

Fig. 4. Haplotype determination using double ARMS The figure illustrates the haplotyping of an individual who is doubly heterozygous at both polymorphic sites 1 and 2. a, b, c, and d are the complimentary bases corresponding to A, B, C, and D, respectively. The object of the double ARMS analysis is to determine whether allele A is linked with allele B or allele A is linked with allele D. This issue can be resolved relatively easily using double ARMS by carrying out amplification using ARMS-A and ARMS-b. If an amplified product is observed, then allele A is linked to allele B on the same chromosome. A second confirmatory PCR can be carried out using ARMS-A and ARMS-d, which should be negative. For a more detailed analysis of the number of ARMS reactions to resolve haplotypes, *see* Lo et al. ***(8)***.

produced. Double ARMS has been shown to be able to detect a single target molecule in an excess of 10^5 background molecules ***(8)***. In this context, double ARMS has been applied to the study of the phenomenon of chimerism following bone marrow transplantation ***(12)*** and in the detection of fetal cells in maternal blood ***(13)***.

Primer design is the most important aspect in creating a working ARMS-based typing system. The ARMS concept requires that the nucleotide or nucleotides distinguishing the various alleles to be placed at the 3' end of the ARMS primer ***(1)***. Different authors have slightly different views as to the type of mismatches that are most discriminatory for ARMS analysis ***(1–3,14,15)***. The reason for this discrepancy between the observations of different groups is unclear but could be related to differences in reaction conditions used by different investigators. In our hands, A-G or G-A mismatches are the most discriminatory for ARMS analysis. For other types of nucleotide mismatches, we routinely introduce an artificial mismatch at the residue 1 or 2 bases from the 3' end of the primer to further enhance the specificity of the primer ***(1)***. Primers ranging from 14 to 30 bases have been used for ARMS analysis ***(1,3,14)***. Newton et al. recommended the use of relatively long primers of 30 bases for this purpose and we have found that primers of this length work well. For a given polymorphism or mutation, an ARMS primer can be designed to prime in the sense or the antisense direction. Hence, if a given ARMS primer is found to be nonspecific in one direction, it is worthwhile constructing another one in the opposite direction.

Allele-specificity is dependent on the annealing temperature during amplification. We routinely start our optimization with an annealing temperature of 55°C and vary the temperature in 3°C steps. Although optimization of the $MgCl_2$ concentration is also important in certain primer combinations, we find that a concentration of 1.5 m*M* works well for most of our applications. The hot start technique ***(1,16)*** is useful for certain "problematic" primers, but for most primer-template combinations, conventional non-hot start PCR appears to be adequate. ARMS specificity is also affected by dNTP concentrations. Hence, a low deoxynucleoside triphosphate (dNTP) concentration has been found to result in a more specific reaction, although sometimes with reduced sensitivity ***(14)***. We find that a dNTP concentration of 100 μ*M* for each of dATP, dCTP, dGTP, and dTTP works well for most ARMS primers.

2. Materials

1. A thermal cycler is necessary for PCR. The author uses either a Biometra TRIO thermoblock or a Perkin-Elmer Cetus (Foster City, CA) DNA Thermal Cycler.
2. Reagents for PCR, including the *Taq* DNA polymerase, dNTPs, and PCR buffer are obtained from a GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer)

Table 1
Primer Sequence

DB3	5'GAC CAC GTT TCT TGG AGC T3'
P-a	5'CTC CTG GTT ATG GAA GTA TCT GTC CAC GT3'
P-b	5'GTC CTT CTG GCT GTT CCA GTA CTC GGA AT3'
P-c	5'CTC CCC CAC GTC GCT GTC GAA GCG CAC GG3'
AAT-1	5'CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG3'
AAT-2	5'GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG3'

3. Oligonucleotide primers for ARMS analysis are available from Genosys (Cambridge, UK) (*see* **Note 1**).
4. DNA samples (*see* **Note 4**).

3. Methods

Multiplex ARMS analysis of HLA DRw52 alleles (*see* **Note 1**).

1. Prepare PCR mix including:
   a. 10X PCR reaction mix.
   b. PCR primers, including an ARMS primer (or a combination of ARMS primers for multiplex ARMS), a downstream non-ARMS primer and internal control primers (*see* **Note 2**). For multiplex ARMS, the relative concentrations of the ARMS primers will need to be optimized empirically (*see* **Note 3**). For the DRw52 system the primer concentrations are: DB3, P-a, and P-b at 1 μ*M*; P-c, AAT-1, and AAT-2 at 0 2 μ*M*
   c. dNTPs typically 100 μ*M* each of dATP, dCTP, dGTP, and dTTP
   d. 1 μg Genomic DNA (*see* **Note 4**).
   e. Sterile distilled water (SDW) to make up to 100 μL.
   f. 2.5 U *Taq* polymerase (*see* **Note 6** and **refs.** *17* and *18*).
   g. 100 μL mineral oil
2. Denature at 94°C for 8 min.
3. Carry out thermal cycling, e.g , 30 cycles of thermal denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and extension at 72°C for 1 min.
4. Perform a final incubation at 72°C for 8 min following the last PCR cycle
5. Analyze 10 μL of the reaction on a 1.5% agarose gel stained with ethidium bromide (*see* **Note 5**).

4. Notes

1. The designing aspects of an ARMS-based system is the most important part for setting up a working assay. Once the design and optimization of amplification conditions have been achieved, the execution of ARMS genotyping is relatively straightforward, analogous to that of conventional PCR.
   In the example, a multiplex ARMS assay is designed to differentiate three groups of alleles at the HLA DRB3 locus *(5)* The three groups of alleles are HLA DRw52a, b, and c. P-a, P-b and P-c are ARMS primers designed to specifically

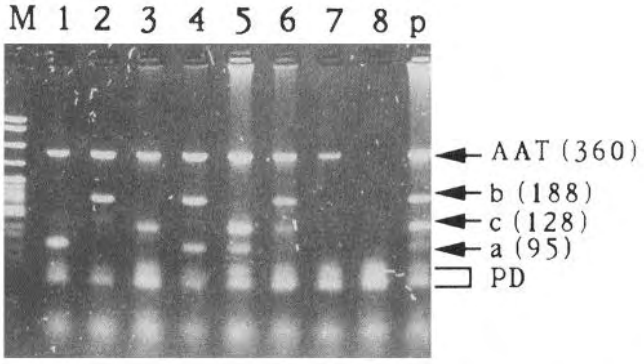

Fig. 5. HLA typing by multiplex ARMS. (**A**) Relative locations of the primers are shown. P-a, P-b, and P-c are ARMS primers designed to specifically amplify DRw52a, b, and c, respectively. DB3 is an upstream primer common to all the alleles. Primer sequences are listed in **Table 1**. (**B**) The results of multiplex ARMS typing are shown. Lane 1, PCR product from individual with DRw52a; lane 2, DRw52b; lane 3, DRw52c; lane 4, DRw52a/b heterozygote; lane 5, DRw52a/c heterozygote; lane 6, DRw52b/c heterozygote; lane 7, no DRB3 alleles; and lane 8, negative control (water). Lane M, pBR322 DNA digested with *Msp*I (marker); Lane P, PCR product ladder. AAT, a, b, and c indicate positions of α-1-antitrypsin positive control, PCR products from the DRB3*0101 (DRw52a), DRB3*0201/2 (DRw52b), and DRB3*0301 (DRw52c), respectively. Numbers in parentheses indicate sizes of PCR products in bp. PD denotes primer dimers. Reprinted with permission from **ref. 5**.

amplify DRw52a, b, and c, respectively. DB3 is an upstream primer common to all the alleles. Primer sequences are listed in **Table 1**. The system is designed such that the three ARMS products have different sizes and thus are distinguishable from each other by gel electrophoresis (**Fig. 5**).

2. As the absence of amplification in an ARMS reaction is used to exclude the presence of a specific allele, most authors include an internal positive control to guard against amplification failures. In our laboratory, we routinely optimize the conditions for an ARMS primer first without the internal control primers. The latter are introduced after the parameters for ARMS analysis have been established.
3. As discussed above, multiplex ARMS is developed in order to simplify the analysis of multiallelic systems so that fewer reactions will be required. All the primers for multiplex ARMS should be designed so that they will exhibit allele-specificity at the same annealing temperature. Furthermore, if different primers have different amplification efficiencies, these have to be balanced by adjusting the respective primer concentrations *(5)*.
4. ARMS specificity is a relative, rather than absolute phenomenon. Hence, the mismatched template will also be amplified, albeit with a much lower efficiency compared with the matched one. There exists a "window" of specificity beyond

which an ARMS system may give rise to a false-positive result. For example, an ARMS primer that exhibits allele-specificity at 25 cycles may no longer be specific at 40 cycles of PCR. Consequently, the amount of DNA template used should be relatively constant for reproducible ARMS-based typing. As a guideline, it is reasonable to optimize ARMS conditions using 1 μg genomic DNA and 30 cycles of PCR. For sample sources in which the yield of amplifiable DNA is less predictable, e.g., paraffin wax embedded archival materials, ARMS analysis may not be as robust as some of the other genotyping methods, e.g., the artificial restriction fragment-length polymorphism method (*see* Chapter 8). If ARMS is intended to be used for DNA extracted from these sources, reproducibility may be improved by using a two-step procedure: using an initial "amplification phase" aiming at amplifying a specific DNA segment from the material and a second 'diagnostic phase' in which a predetermined amount of the first phase PCR product is used for ARMS analysis *(17, 18)*. The amplification phase PCR product should be diluted by a specified amount (typically 10^5 times) before being subject to the diagnostic ARMS amplification.

5. To facilitate the reading of the results of a multiplex ARMS genotyping, a "PCR-product ladder," consisting of all the possible PCR products, can be included on the gel
6. Specificity can also be increased by decreasing the primer concentration from the usual 1 μ*M* to 0.05–0.25 μ*M (2)*. Further increase in specificity can be achieved by decreasing the concentration of *Taq* from 2.5 to 1 U
7. When using ARMS or double ARMS to detect a minority DNA population, the inclusion of an internal amplification control is not desirable. This is because the internal control primers will amplify the control sequence in the majority DNA background; once the internal control PCR product has reached the amplification plateau, the ARMS product yield will be greatly diminished. A better positive control is to run a parallel dilution series with known amount of the target DNA molecules.

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8

Artificial Restriction Fragment Length Polymorphism (A-RFLP) Analysis

Y. M. Dennis Lo and Virginia A. Horton

1. Introduction

Restriction analysis of polymerase chain reaction (PCR) products is one of the earliest techniques used for analyzing amplification products *(1)*. This approach is applicable for distinguishing alleles in which the polymorphic residue results in the creation or removal of a restriction enzyme site. Unfortunately, many polymorphisms are not associated with restriction enzyme site change and thus are not amenable to this analysis. However, by using site-directed mutagenesis using primers with mismatches near the 3' ends, it is possible to create an artificial restriction fragment length polymorphism (A-RFLP) for almost all naturally occurring DNA polymorphisms *(2–5)*. **Figure 1** illustrates the principles of this approach.

Design of an A-RFLP primer can be accomplished easily and rapidly using a semiautomatic approach using a computer program that will search for restriction enzyme sites for a given sequence, e.g., DNA Strider. The process is illustrated in **Fig. 2**. For the following discussion, let us assume that the polymorphic residue is P (**Fig. 2**) and that we are searching for restriction enzymes with recognition sites of up to six bases. Five bases on either side of P are entered into the computer (from –5 to +5) and the program is used to search for a restriction enzyme site encompassing P. If a restriction enzyme site is found that is only present in one allele but not in the other one, then an A-RFLP site is found. If no restriction site polymorphism is found, then the nucleotides from –2 to –5 and from +2 to +5 are systematically changed one at a time, and a computer-assisted search for restriction enzyme sites is carried out after each alteration. For each position, the nucleotides A, T, C, and G is substituted in turn (one of them will be found in the naturally occurring sequence).

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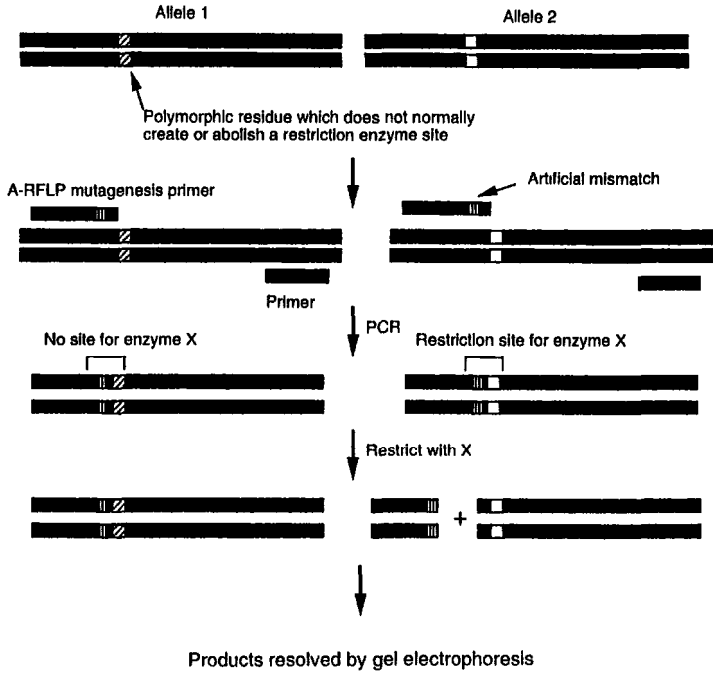

Fig. 1. Principles of artificial RFLP. Reprinted with permission from **ref. *6***.

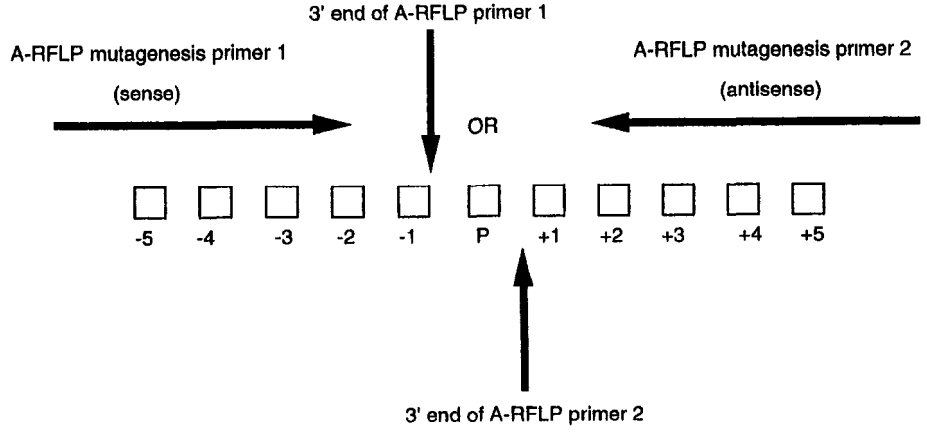

Fig. 2. Semiautomatic approach for designing A-RFLP primers. See text for details. Following the design stage, only one of the A-RFLP primers would be chosen, either in direction 1 (sense) or direction 2 (antisense). PCR amplification would then be carried out using the A-RFLP primer and a downstream primer. Reprinted with permission from **ref. *6***.

We avoid changing the –1 or the +1 position (which will be used as the last base of the PCR primer) as this may reduce the amplification efficiency. It is sometimes worthwhile to carry on the process until all the possibilities have been investigated, as more than one solution may be possible for a given polymorphism and some restriction enzymes work better than others.

Allelic assignment by A-RFLP is a two-step process, with an initial amplification step, followed by a diagnostic restriction step. PCR amplification with A-RFLP primers is carried out as for conventional PCR. Restriction endonuclease digestion is carried out following DNA amplification. Following endonuclease restriction, the PCR product from the allele with the restriction site will have the portion containing the A-RFLP primer cleaved off, thus resulting in a fragment of a smaller size on agarose gel electrophoresis. We typically use A-RFLP primers, which are approx 30 bases in length. The optimal size of the PCR product is approx 120 bp. Agarose gels of 1.5% normally give adequate resolution for allelic discrimination.

2. Materials

1. A thermal cycler is necessary for PCR. The authors use either a Biometra TRIO thermoblock or a Perkin-Elmer Cetus DNA Thermal Cycler (Foster City, CA).
2. Reagents for PCR, including the *Thermus aquaticus* (*Taq*) DNA polymerase, deoxynucleoside triphosphates (dNTPs), and PCR buffer are obtained from a GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer).
3. Oligonucleotide primers for A-RFLP analysis were obtained from Genosys (Cambridge, UK) (*see* **Note 3**).
4. Restriction enzymes and restriction buffers (commercially available).
5. DNA samples (*see* **Notes 4** and **7**).
6. Agarose gel electrophoresis equipment and gel documentation system.

3. Methods

3.1. Clinical Application 1: Aspartate-57 Status of the HLA DQB1 Gene

The HLA DQB1 locus represents a multiallelic system with 17 alleles. These alleles could be divided into those coding for aspartic acid at codon 57 and those that do not. The aspartic acid status at codon 57 is important for determining susceptibility and resistance to insulin-dependent diabetes mellitus (IDDM) *(7)*. The various DQB1 alleles coding for aspartic acid at position 57 have in common GA as the first two nucleotides of the codon (**Fig. 3**). Codon 58 is GCC in all alleles. Thus, a *Hinf*I site (GANTC) diagnostic for the aspartate-57 alleles could be created by mutating codon 58 from GCC to TCC via appropriately constructed mutagenesis primers. As the third base of codon 57 could be C or T, two PCR primers would be necessary to direct amplification for these two groups of alleles. Primer sequences are listed in **Table 1**. An amplification product of 199 bp is formed.

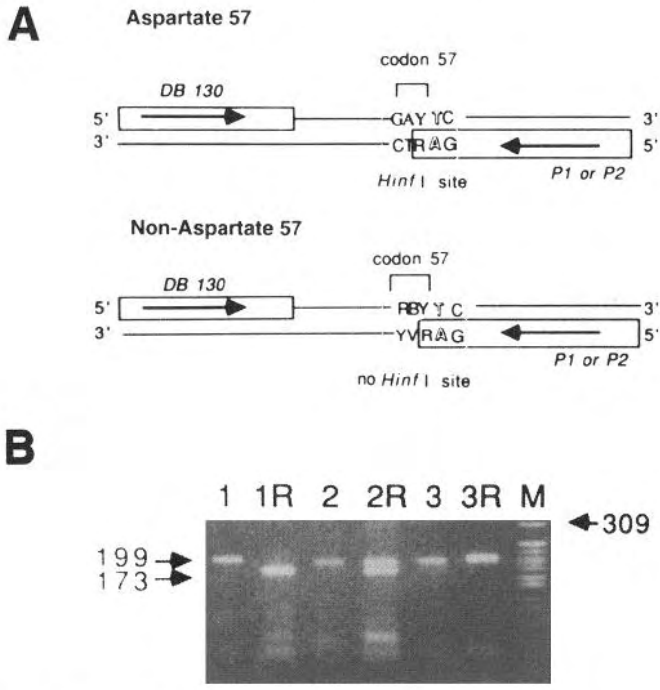

Fig. 3. A-RFLP analysis for aspartate-57 status of the DQB1 gene. (**A**) Diagram illustrating the principle of A-RFLP primer design. P1 and P2 are the A-RFLP mutagenesis primers. DB130 is an upstream primer common to all DQB1 alleles ***(12)***. The penultimate nucleotide A of primers P1 and P2 is an artificial mismatch. Y = T or C; R = A or G; B = T, G, or C; V = A, C, or G (IUPAC nomenclature). (**B**) Typing of aspartate-57 status at the DQB1 gene by A-RFLP. 1, 2, and 3 represent undigested PCR products from three individuals. 1R, 2R, and 3R represent the PCR products digested with *Hinf*1. Individual 1, aspartate-57 homozygote; individual 2, aspartate-57/nonaspartate-57 heterozygote; and individual 3, nonaspartate-57 homozygote. Lane M, pBR322 DNA digested with *Msp*1 (marker). 199, uncut PCR product in bp; 173, cut PCR product in bp; 309, 309 bp band of the size marker. Reprinted with permission from **ref. *8***.

By using this A-RFLP system, the 17 alleles of the DQB1 gene can be divided into those with aspartate-57 and those without aspartate-57 in a simple system using PCR followed by restriction with the enzyme *Hinf*I.

1. Prepare PCR mix including
   a. 10X PCR reaction mix.
   b. PCR primers (P1, P2, and DB130), typically 100 pmol per 100 µL reaction.
   c. dNTPs typically 100 m*M* each of dATP, dCTP, dGTP, and dTTP.
   d. 1 µg genomic DNA.

Table 1
Primer Sequences[a]

P1	5'TTC CTT CTG GCT GTT CCA GTA CTC GG*A* G3'
P2	5'TTC CTT CTG GCT GTT CCA GTA CTC GG*A* A3'
DB130	5'AGG GAT CCC CGC AGA GGA TTT CGT GTA CC3'
DR3 A-RFLP	5'CCG CTG CAC TGT GAA GCT CTC CAC AAC CCC GTA GTT GTG TCT GCA *C*TA G3'
DR4.04	5'CGG GTG CGG TTC CTG GAC AGA TAC TTC *G*AT3'

[a]Deliberate mismatches are in italics

e. Sterile distilled water (SDW) to make up to 100 µL.
f $MgCl_2$ at 1.5 m*M*
g 2.5 U *Taq* polymerase
h. 100 µL mineral oil

2. Denature at 94°C for 8 min
3. Carry out thermal cycling, 30 cycles of thermal denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min (conditions using a Biometra TRIO thermoblock) (*see* **Note 5**)
4. Perform a final incubation at 72°C for 8 min following the last PCR cycle.
5. Set up restriction mixture consisting of
   a 10 µL PCR product.
   b 7 µL SDW
   c. 2 µL 10X Restriction buffer (provided by enzyme manufacturer)
   d 1 µL *Hinf*1 Restriction enzyme (20–60 U) (*see* **Note 1**).
6. Restrict for 4 h at 37°C.
7. Analyze 10 µL of the restricted PCR product in a 2% agarose gel stained with ethidium bromide (*see* **Note 2**).

3.2. Clinical Application 2: HLA DR3 and DR4 Typing

A-RFLP has also been applied to the typing of the much more complex HLA DRB1 gene in which there are 59 known alleles divided into 11 allelic subgroups. Two of these allelic subgroups, DR3 and DR4, which consist of 3 and 12 alleles, respectively, are highly associated with IDDM with DR3/DR4 heterozygotes being at particularly high risk *(9)*. Reliable identification of DR3 and DR4 alleles in susceptible populations is helpful in identifying subjects who may be at risk of developing IDDM and assists in determining the role of these alleles in the etiology and pathogenesis of IDDM. In the following example, an A-RFLP approach is developed to detect DR3 and DR4 *(10)*. Thus, by the use of A-RFLP mutagenesis primers, *Sau*3A 1 and *Spe*1 sites are created to identify DR4 and DR3 alleles, respectively. Primer sequences are listed in **Table 1**. The primers are in regions that are highly conserved among the various alleles, thus they are able to amplify all or at least most alleles tested. An amplification

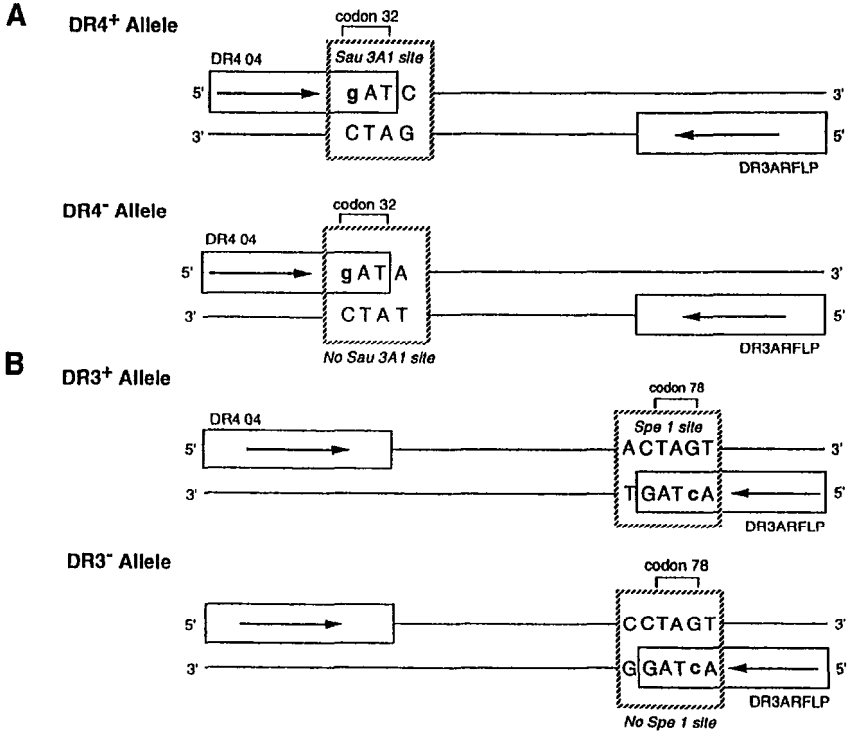

Fig. 4. Diagrams illustrating the principles of A-RFLP for typing the DR3 and DR4 alleles of the DRB1 gene. (**A**) DR 4 detection. A deliberate mismatch of C to G at codon 32 is introduced in the 3' end of the primer (lower case g). *Sau*3A 1 cuts the resulting sequence GATC, which is only present in the DR4 PCR product. (**B**) DR3 detection A deliberate mismatch of G to C at codon 78 is introduced in the 3' end of the primer (lower case c). *Spe*1 cuts the resulting sequence ACTAGT, which is only present in DR3 and in the DRB7 pseudogene PCR products. Reprinted with permission from **ref. *10***.

product of 213 bp is formed. The two primers are designed to be of significantly different sizes (49 bases for DR3 A-RFLP and 30 bases for DR4.04) so that primers with one of the other primers cleaved off can be distinguished on gel electrophoresis. Furthermore, it should be noted that a *Spe*1 site is also created on amplification of DRB7, a pseudogene that is in linkage disequilibrium with certain DRB1 alleles, including DR4. Thus an additional enzyme, *Dde*1, that recognizes a CTNAG site in the DRB7 pseudogene not present in DR3 alleles, is used to differentiate DRB7 from DR3.

Thus, digestion by *Sau*3A 1 of the GATC sequence that only occurs in DR4 alleles gives a diagnostic 186-bp fragment (formed by the cleavage of the DR4.04 primer) (**Fig. 4**). Digestion of the ACTAGT sequence that only occurs

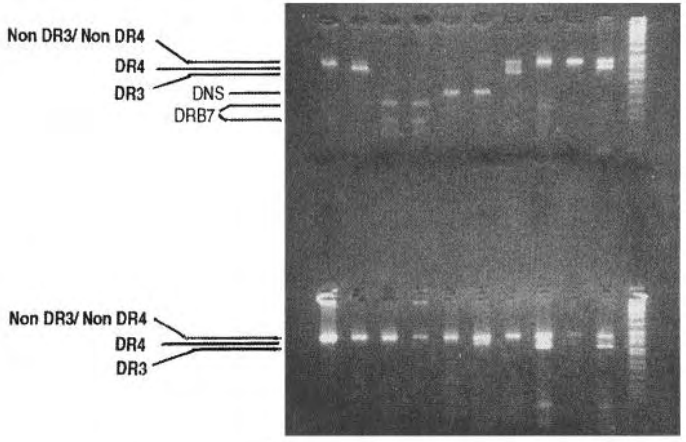

Fig. 5. DR3 and DR4 typing using A-RFLP. PCR products from individuals with DR4, DR3, DR3/DR4, DRB7, a *Dde*1 positive but non-DRB7 site (DNS), and without any of the above (N). The upper part of the gel shows N, DR4, DRB7, DRB7, DNS, DNS, DR3/DR4, N, N, DR4, and pBR322 DNA cut with *Msp*1 (marker); the lower part of the gel shows N, N, N, N, N, DR4, N, DR3/DR4, N, DR3, and pBR322 DNA cut with *Msp*1 (marker). Reprinted with permission from **ref. *10***.

in the DR3 or DRB7 alleles by *Spe*1 gives a 164-bp fragment (formed by the cleavage of the DR3 A-RFLP primer) (**Fig. 4**). The restriction enzyme *Dde*1 differentiates between the alleles because it recognizes two CTNAG sites in DRB7, at codons 51–52 and 58–59, and none in DR3. On digestion of a DRB7 allele with *Dde*1, the PCR product is cut into three fragments: 106, 86, and 21 bp, although the latter is too small to be detected reliably. In samples positive for alleles containing a *Dde*1 site other than the DRB7 allele, the second cutting site is absent so that a 106/107-bp doublet is observed. Representative results are shown in **Fig. 5.**

1. Prepare PCR mix including:
   a. 10X PCR reaction mix.
   b. PCR primers (DR3 A-RFLP and DR4.04), typically 50 pmol per 50 µL reaction.
   c. dNTPs: typically 100 µ*M* each of dATP, dCTP, dGTP, and dTTP.
   d. 0.1–1 µg genomic DNA.
   e. SDW to make up to 100 µL.
   f. 2 m*M* $MgCl_2$.
   g. 2 U *Taq* polymerase.
   h. 50 µL mineral oil (omit if Perkin Elmer 9600 thermal cyclers are used with heated lids).
2. Denature at 94°C for 8 min.
3. Carry out thermal cycling, 35 cycles of thermal denaturation at 95°C for 20 s,

primer annealing at 55°C for 20 s and extension at 72°C for 30 s (times as for Perkin Elmer 9600 thermal cycler).

4. Perform a final incubation at 72°C for 8 min following the last PCR cycle.
5. Set up restriction mixture consisting of:
   a 12 µL PCR product.
   b 1.5 µL 10X Restriction buffer (provided by enzyme manufacturer)
   c 1 µL *Spe*1 restriction enzyme (20 U)
   d 1 µL *Dde*1 restriction enzyme (20 U)
6 Restrict for 2 h at 37°C.
7 12 µL of first restriction mixture is then restricted using *Sau*3A 1
   a 12 µL product previously cut with *Spe*1 and *Dde*1
   b. 1.5 µL 10X restriction buffer (provided by enzyme manufacturer)
   c 1 µL *Sau*3A 1 restriction enzyme (20 U)
   d 0 5 µL SDW
8. Restrict for 2 h at 37°C
9. Analyze 10 µL of the restricted PCR product in a 3.5% metaphor agarose gel (FMS Bioproducts, Rockland, MD) stained with ethidium bromide (*see* **Note 6**)

4. Notes

1 Concentrated preparations of restriction enzymes (>40 U/µL) have been found to give the best results
2 During the electrophoresis stage, it would be useful to include a lane containing the unrestricted PCR product for comparison.
3. In constructing A-RFLP systems for mutation detection, it would be more robust to design the system such that the presence of the mutation is detected by the creation of a restriction site, rather than the reverse when the existence of the mutation abolishes a restriction site. This is because although it is relatively straightforward to deduce that the presence of a smaller DNA fragment following restriction analysis signifies the presence of the restriction site, the absence of the smaller product may be due to either the absence of the site or the failure of restriction. Furthermore, partial restriction, which is not unusual, may give a false diagnosis of heterozygosity.
4 A-RFLP can also be used to detect a minority DNA population *(11)* i.e., when the target DNA molecules only constitute a minor proportion of the total DNA sample
5 It is important not to carry the PCR to an unnecessarily high cycle number due to the theoretical possibility of producing heteroduplexes in heterozygous individuals. These are DNA molecules formed by the annealing of DNA strands amplified from the two alleles of a heterozygous individual. Heteroduplexes are refractory to restriction.
6. For the DR3/DR4 typing system, electrophoresis using a 3 5% metaphor agarose gel (FMS Bioproducts) has been found to improve resolution.
7. A-RFLP is generally a very robust method. Compared with the amplification refractory mutation system (ARMS) (*see* Chapter 7), it has the advantage of separating the amplification and the diagnostic steps, which in ARMS is combined

into a single step Hence, in an A-RFLP system, there is always a control for potential amplification failure, which in ARMS, the absence of an amplified product may be interpreted erroneously as indicative of the absence of the mutation concerned. Its inherent robustness makes A-RFLP most useful for the analysis of archival materials, e.g , paraffin wax-embedded materials, when the yield of amplifiable materials may be variable ***(10)***.

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9

Long Range PCR

William Waggott

1. Introduction

1.1. Background to Long Range PCR Amplification

Conventional polymerase chain reaction (PCR) enables reliable amplification of 3–4 kb of DNA *(1)* while attempts at optimisation has enabled 15.6 kb of λ DNA to be amplified *(2)*. The maximum amplifiable length of PCR is limited by the low fidelity of the *Thermus aquaticus* (*Taq*) DNA polymerase *(3)*, the most commonly used thermostable polymerase. It is believed that inadvertent nucleotide misincorporations during the PCR extension steps cause chain terminations *(3)*. The *Taq* polymerase lacks proofreading properties *(4)* and thus is unable to correct such misincorporations. The higher extension K_M value for a misincorporated nucleotide is thought to cause detachment of the *Taq* polymerase from template DNA.

The turning point in the development of long and accurate PCR amplifications has been the discovery that proofreading enzymes with exonuclease activity enables a ten-fold increase in the PCR product length. Utilizing λ bacteriophage DNA as a template, Barnes first reported the high fidelity amplification of 35 kb of DNA *(5)*. A proofreading enzyme with 3'–5' exonuclease activity, *Pyrococcus furiosus (Pfu)* DNA polymerase, was used in conjunction with *Taq*. This record was later exceeded by the amplification of 44 kb of lambda DNA and a 22 kb fragment from human genomic DNA *(6)* using similar methodology. It is believed that the *Pfu* polymerase circumvents the low fidelity of the *Taq* polymerase by correcting the nucleotide misincorporations, paving the way for the *Taq* polymerase to complete the polymerization reaction.

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Table 1
A Selection of Reports Utilizing Long Range PCR

Ref.	Target	Cloning and characterization
18	Hepatitis B genome	Structural characterization of heterogeneous populations of complete virion-encapsidated full length HBV DNAs from sera of transplant patients
19	Mouse L-isoaspartyl/D-aspartyl methyl-transferase gene	Determined the exon positions of methyl-transferase gene by restriction mapping of long PCR fragments
20	Long inserts cloned on lambda EMBL3 phage vector	Rapid mapping of the restriction sites of long inserts cloned in lambda EMBL3 phage vector
21	Retinoblastoma gene (RB1)	Coamplification of 24 RB gene exons prior to multiplex mutational scanning by two-dimensional denaturing gradient gel electrophoresis (DGGE)
		Clinical diagnosis
14,15	Nucleophosmin-Anaplastic lymphoma kinase gene fusion	Gene fusion at the t(2;5)(p23,q35) breakpoint, a diagnostic gene rearrangement for a type of non-Hodgkin's Lymphoma
7	Human polyomavirus JC (JCV)	PCR from clinical specimens of progressive multifocal leukoencephalopathy patients prior to RFLP analysis and sequencing for viral typing and characterization
16	Various gene fusions found at translocation breakpoints	Amplification of junctional sequences in chromosome translocations of B cell neoplasms
11	Trinucleotide repeat expansion	CTG trinucleotide repeat expansions associated with myotonic dystrophy was PCR amplified to facilitate molecular diagnosis and studies of the disease

1.2. Utility of Long Range PCR

Long accurate amplification has enhanced the utility of PCR in genetic and clinical studies. A selection of research reports utilizing long range PCR are listed in **Table 1**.

In gene isolation, long range PCR would minimize laborious microbiological and vector manipulation procedures by enabling direct isolation of cDNA inserts. A 50-kb insert may be amplified in two halves starting from the flanking vector sequences. Studies into gene structure would be assisted by enabling amplifications across intron/exon boundaries. PCR-based chromosome walking would also be greatly enhanced.

Whole viral *(7)* and mitochondrial *(8,9)* genomes have been amplified to search for gene deletions and mutations. Amplification of FRDA gene intron in Friedreich's ataxia *(10)* has shown that the intronic GAA triplet expansion is a potential pathogenic pathway. Presymptomatic disease diagnosis may thus be possible in this and other diseases sharing similar modes of pathogenesis such as Huntington's disease and myotonic dystrophy *(11)*.

Recent development of a long range reverse transcriptase PCR (RT-PCR) system has extended the scope for assaying for rare transcripts and tissue specific gene expression. Amplifications of full length transcripts of hepatitis A virus *(12)* and transcripts as large as 13.5 kb has recently been demonstrated. Long range PCR has also impacted on the differential display technique, allowing larger transcripts to be studied *(13)*.

Detection of gene rearrangements found at chromosome translocation breakpoints in hematological malignancies may be greatly facilitated by long range PCR and may be helpful in clinical diagnosis. Molecular diagnosis of such abnormality usually relies on RT-PCR or Northern blotting for the detection of novel chimeric transcripts resulting from gene fusion. At the DNA level, such gene rearrangements span distances beyond the amplification limit of conventional PCR and thus can only be analyzed by Southern blotting. Thus, in designing a genomic DNA-based PCR detection system for a recently characterized nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) gene fusion, of unknown intron structure, we *(14)* and others *(15)* have adopted the long range PCR methodology. Long range PCR has also been utilized in the detection of other gene rearrangements *(16)*.

1.3. Model Systems: Detection of NPM-ALK Gene Fusion of t(2;5)(p23;q35) Chromosomal Abnormality in Anaplastic Large Cell Lymphoma (ALCL) and Amplification of 23 kb of Genomic DNA

In this chapter, application of long range PCR to the detection of NPM-ALK gene fusion from genomic DNA is outlined *(14)* utilizing an Expand™ Long Template PCR System (Boehringer Mannheim) (*see* **Table 2** and **Note 1**). The NPM-ALK gene fusion was recently identified at the genomic breakpoint of the t(2;5)(p23;q35) chromosome translocation, found in anaplastic large cell lymphoma *(17)*. In order to further characterize the intronic region of the translocation breakpoint in seven cases of ALCL, a long range PCR strategy was adopted. In a second example, a 23-kb fragment of the human β-globin gene region is amplified from genomic DNA using a 20 kb Plus System (Boehringer Mannheim, Mannheim, Germany).

Table 2
Representative Long Range PCR Kits and Notable Features

Product name	Manufacturer	Proofreading enzyme	Second polymerase enzyme	Ref
Expand™ Long Template PCR System	Boehringer Mannheim	*Pwo* 3'–5' exonuclease	*Taq*	***18,24***
TaKaRa LA PCR™ Kit Version 2	TaKaRa	3'–5' exonuclease	LA*Taq*	***16,25***
Gene Amp XL PCR Kit	Perkin-Elmer	Vent 3'–5' exonuclease	r*Tth*	***10,26***
Taq Plus™	Stratagene	*Pfu* 3'–5' exonuclease	*Taq*	***27***
Elongase	Life-Technologies	GD-B *Pyrococcus* 3'–5' exonuclease	*Taq*	not available

2. Materials

2.1. Template DNA Preparation

1 Cultured cell lines SU-DHL-1 and Karpas 299.
2. Human peripheral blood.
3 Nucleon 1 DNA extraction kit (Scotlab, Strathclyde, Scotland, UK).
4. QIAGEN Genomic-tip 20G/100G (Qiagen, Surrey, UK).

2.2. Thermocycling Machine

Thermal cyclers capable of extending the extension time for successive cycles are recommended. Perkin-Elmer Gene Amp 9600/Biometra Trio Thermoblock Thermal Cycler (Norwalk, CT).

2.3. PCR Primers (*see* Note 2)

1. Amplification of NPM-ALK gene fusion.
   a. Forward primer 5' NPM (100 ng/μL stock), M1: 5'-TCC CTT GGG GGC TTT GAA ATA ACA CC-3'.
   b. Reverse primer 3' ALK (100 ng/μL stock), M2: 5'-CGA GGT GCG GAG CTT GCT CAG C-3'.

   These PCR primers were designed from the respective exons of the NPM and ALK genes involved in the translocation breakpoint. The position of the PCR primers with respect to the known genomic sequences are illustrated in **Fig. 1A**.
2 Amplification of human β-globin gene.

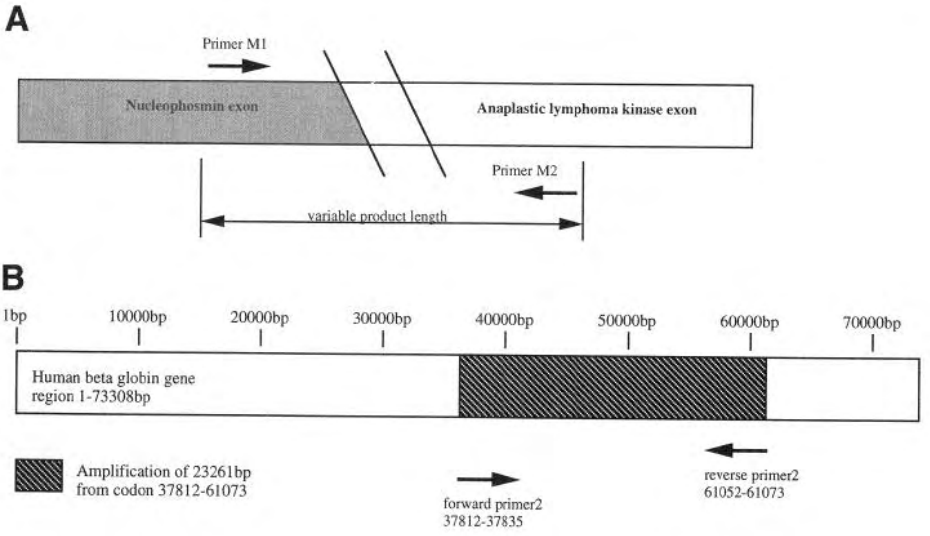

Fig. 1. (**A**). Schematic diagram NPM-ALK gene fusion at t(2;5)(p23;q35). (**B**) Human β-globin gene region on chromosome 11 (DNA sequence source: National Center of Biotechnology Information, NCBI ID:U01317), indicating PCR primer positions and the PCR amplified regions.

a. Forward primer 2 (158 ng/μL stock), 5'-CAC AAG GGC TAC TGG TTG CCG ATT-3'.
b. Reverse primer 2 (145 ng/μL stock), 5'-AGC TTC CCA ACG TGA TCG CCT T-3'.

The locations of these primers are indicated in **Fig. 1B**.

2.4. PCR Reaction Tubes

Thin-walled 0.2 or 0.5-mL reaction tubes may be used. Thin-walled tubes ensure efficient heat exchange between the thermoblock and the reactions.

2.5. Long Range PCR Systems

1. Expand Long Template PCR System (Boehringer Mannheim).
2. Expand 20 kb Plus System (Boehringer Mannheim).

3. Methods

3.1. Template DNA Preparation

1. Extract DNA for cultured cell lines SU-DHL-1 and Karpas 299 cell lines with t(2;5)(p23;q35) chromosome translocation using a Nucleon 1 DNA extraction kit.
2. Use normal human peripheral blood as a control.

Table 3
Composition of Reaction Mixes

Reaction components (stock concentrations)	Volume (μL) NPM-ALK Expand™ Long Template	Volume (μL) β-Globin 20 kb Plus System
Master mix 1		
dNTPs, 100 m*M* nucleotide mix	1 0	1.0
Forward primers	1 0	1.0
5' NPM;100 ng/μL stock primer M1		
5' β-globin;158 ng/μL stock: forward primer 2		
Reverse primers	1.0	1 0
3' ALK;100 ng/μL stock primer M2		
3' β-globin;145 ng/μL stock reverse primer 2		
0 2 mg/μL human genomic DNA	1 0	1 0
Sterile redistilled water	23 0	23 0
Final volume	25.0	25.0
Master mix 2		
PCR buffer mix	5.0[a]	10.0
Enzyme mix	0 75	0 75
Sterile double-distilled water	19.25	14.25
Final volume	25.0	25.0

[a]*See* **Note 4**

3.2. Long Range PCR Methodology

1. Thaw the reaction components and place on ice
2. Prepare two separate reaction mixes as outlined in **Table 3**, separating the primer, template, and nucleotides from the enzyme and buffer mix This is important for long range PCR using DNA polymerase with a 3'–5' exonuclease activity, e.g , *Pwo*, which would otherwise digest the free primers
3. Gently agitate the reaction mixes to thoroughly mix the components before combining the two halves. Mix thoroughly again and centrifuge briefly to collect the solution
4. Overlay 30 μL of paraffin oil
5 Place tubes on PCR machine and start thermocycling immediately

3.3. Thermocycling Profiles and Other Variables

1. Thermocycling profiles are adapted to suit the size of the target DNA template. For amplification of 23 kb of genomic DNA, an extension time of 18 min is required

2. The concentration of dNTP, $MgCl_2$, template, and enzyme concentrations are modified according to the template size and whether the template is genomic DNA or λ DNA. These parameters should be adjusted as outlined in the manufacturers' instructions.

3.4. Thermocycling Profile

1 Amplification of the NPM-ALK gene fusion product from genomic DNA samples of ALCL cases
PCR conditions were adapted for the amplification of up to 5 kb of genomic DNA
a Initial denaturation at 92°C for 2 0 min.
b Denature 92°C for 10 s; anneal 65°C for 30 s; elongation 68°C for 45 s for a total of 10 cycles.
c Denature 92°C for 10 s, anneal 65°C for 30 s, elongation 68°C for 45 s for a total of 10 cycles.
d Denature 92°C for 10 s; anneal 65°C for 30 s; elongation 68°C for 45 s, increase elongation time by 20 s increments in each successive cycle for a total of 10 cycles.
e Final extension time at 68°C for 7 min
Store at 4°C until analysis by agarose gel electrophoresis.

2. Amplification of β-globin gene from human genomic DNA.
PCR conditions were adapted for the amplification of up to 20–35 kb of genomic DNA.
a. Initial denaturation at 92°C for 2.0 min.
b. Denature 92°C for 10 s; anneal 65°C for 30 s; elongation 68°C for 45 s for a total of 10 cycles
c Denature 92°C for 10 s; anneal 62°C for 30 s, elongation 68°C for 18 min, increase elongation time by 20 s increments in each successive cycle for 19 cycles for a total of 20 cycles.
d Final extension time at 68°C for 7 min.
Store at 4°C until analysis by agarose gel electrophoresis.

3.5. PCR Product Analysis

1. Mix 15 µL of each PCR reaction products with 3 µL of loading dye.
2. Load onto a 0.8% agarose 1X TBE minigel gel. The long β-globin PCR products can be run at 60 V for 3 h (**Fig. 2A**). For smaller products up to 3.0 kb long an 1% agarose 1X TBE minigel has achieved satisfactory resolution run at 100 V for 60 min (**Fig. 2B**).

4. Notes

1. Methodologies in long range PCR are based in the majority on commercially available kits. A large number of such kits utilize various proofreading enzymes in conjunction with thermostable DNA polymerases Representative commercial products and their notable features are listed in **Table 2** Long amplifications can also be achieved using proofreading enzymes alone Although polymerases with proofreading activity generally have inferior processivity to *Taq* (thus limiting

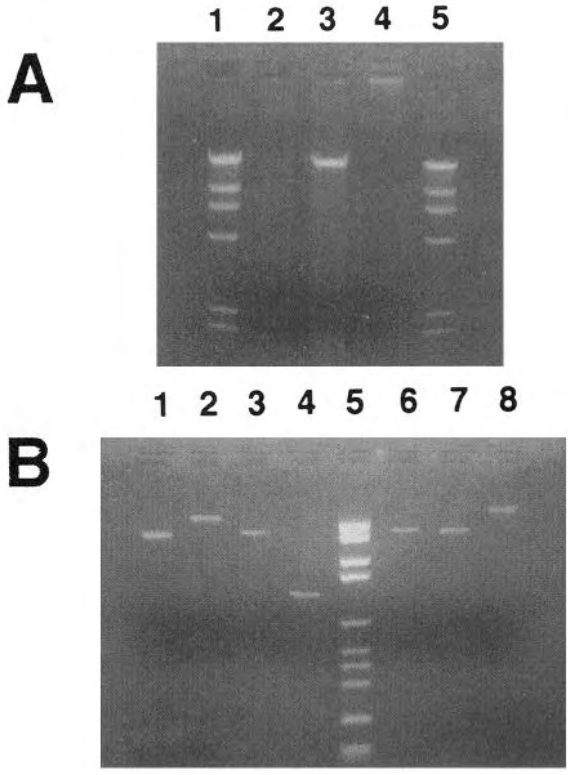

Fig. 2. (**A**) Amplification of 23261 bp of human β-globin gene from genomic DNA. Lanes 1 and 5, DNA size marker lanes, λ DNA digested with *Hind*III, 23,130, 9416, 6557, 4361, 2322, and 2027 bp. Lane 2, β-globin amplification from human genomic DNA extracted using conventional methodology from human peripheral blood. Lane 3, β-globin amplification from human genomic DNA extracted using Qiagen genomic tip for preparation of high molecular weight DNA from human peripheral blood. Lane 4, Negative control. (**B**) Amplification of NPM-ALK gene fusion from genomic DNA. Lanes 1–4 and 6–8: NPM-ALK amplification from genomic DNA from tumor tissue of seven ALCL non-Hodgkin's lymphoma patients. Lane 5, DNA size marker lane, 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, and 234 bp.

their application to products of up to 5 kb), they have better fidelity to *Taq*/proof-reading enzyme combinations ***(22)***. These single enzyme systems are thus recommended for applications in which fidelity is of great importance.

2. PCR primers should ideally have balanced T_m (between 63°C and 68°C) not differing by more than 1°C or 2°C and be between 21–34 nucleotides in length. Care must be taken to avoid opportunities for dimer and secondary structure formation.

3. Extremely long PCR amplifications from genomic DNA (20 kb or longer) requires DNA of high integrity *(23)*. The integrity of DNA may be checked by running the DNA on a 0.3% agarose gel. Single stranded nicks or double stranded breaks caused by manipulation procedures in conventional DNA extraction protocols reduce the copy number of long target sequences and are not suitable for long amplification (**Fig. 2A**).
4. The Expand PCR System offers a choice of three buffer systems to suit the size of the DNA template to be amplified.
   a. Buffer 1: 0 5–12 kb of λ DNA and 0.5–25 kb of genomic DNA.
   b. Buffer 2: 12–15 kb of λ DNA and 25–30 kb of genomic DNA.
   c. Buffer 3: >15 kb of λ DNA and >30 kb of genomic DNA.

   The 20 kb Plus System has a single buffer.

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10

Generation of Labeled Probes by PCR

Y. M. Dennis Lo and Shu F. An

1. Introduction

Nucleic acid probes are an important tool in molecular diagnosis. To facilitate the detection of hybridized probes, they are labeled with a reporter molecule, which is usually a radioisotope. For diagnostic techniques carried out in a clinical laboratory, radioisotopes are hazardous and, thus, recently there is a move to use nonisotopic labels, such as biotin and digoxigenin. Nonisotopic probes also have the advantage of much better stability over time compared with isotopic probes that have limited half-lives.

Conventional techniques using cloned DNA for probe production are time-consuming, involving the preparation of plasmid DNA with the probe DNA as an insert, restriction enzyme digestion followed by preparative gel electrophoresis to separate the probe DNA from the plasmid, and then finally the carrying out of the labeling reaction (typically nick translation *[1]* or random priming *[2]*).

The development of the polymerase chain reaction (PCR) allows an alternative, more efficient method in which probe DNA is amplified and labeled in a single step by the amplification reaction. The most important advance being the demonstration that the *Thermus aquaticus (Taq)* DNA polymerase commonly used in PCR is able to incorporate nucleotide analogs during DNA amplification *(3)*. Nucleotide analogs such as biotin-11-dUTP *(3)* or digoxigenin-11-dUTP *(4)* have been successfully used as substrates for PCR labeling. In this chapter, we present the protocols for using the PCR to generate biotin- and digoxigenin-labeled probes.

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Table 1
Sequence of PCR Primers

Sequence name	Sequence
PCR1	5'GATTGAGATCTTCTGCGACGC3'
PCR2	5'GAGTGTGGATTCGCACTCCTC3'
BB1660	5'CTTGTTGACAAGAATCCTCAC3'
BB1661	5'GATGGGATGGGAATACA3'
BB1666	5'CAGAGTCTAGACTCGTGG3'
BB1667	5'ACAAACGGGCAACATACCTTG3'
BB1671	5'GACATACTTTCCAATCAATAG3'
T3 promoter	5'ATTAACCCTCACTAAAGGGA3'
SP6 promoter	5'GATTTAGGTGACACTATAG3'
T7 promoter	5'TAATACGACTCACTATAGGG3'

2. Materials

1. Reagent buffer, dATP, dCTP, dGTP, dTTP and *Taq* polymerase for PCR from Perkin-Elmer (Foster City, CA)
2. Biotin-11-dUTP in powder form from Sigma (St. Louis, MO), to be dissolved in 100 m*M* Tris-HCl, pH 7.5, 0.1 m*M* edetic acid (EDTA) to make a 0 3 m*M* solution before use.
3. Digoxigenin-11-dUTP (dig-11-dUTP) from Boehringer Mannheim (East Sussex, UK).
4. DNA thermocycler, such as the Biometra TRIO thermoblock.
5. PCR primers from British Biotechnology Ltd (Abingdon, UK). Primer sequences are listed in **Table 1**.
6. Paraffin oil to prevent evaporation during PCR (BDH, Merck Ltd, Dorset, UK)
7. 1XTBS: 0 15 *M* NaCl, 0.015 *M* Tris-HCl, pH 7 2.
8. Blocking buffer. 0.1 *M* Tris-HCl, 0 1 *M* NaCl, 3 m*M* $MgCl_2$, 0 5% Tween-20, pH 7 5.
9. AP7.5: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 3m*M* $MgCl_2$, pH 7 5
10. AP9.0: 0.1 *M* Tris-HCl, 0.1 *M* NaCl, 0.1 *M* $MgCl_2$, pH 9.0.
11. 1X SSC: 0.3 *M* NaCl, 0 03 *M* sodium citrate.
12. Phosphate-buffered saline (PBS): 0.012 *M* Na_2HPO_4, 0.04 *M* KH_2PO_4, 0.15 *M* NaCl, pH 7 2.
13. 1 X SET: 0 15 *M* NaCl, 0.02 *M* Tris-HCl, 1 m*M* EDTA, pH 7 8.

3. Methods

3.1. Incorporation of Biotin-dUTP by PCR

1. Prepare PCR mix. Use TTP and biotin-11-dUTP at a ratio of 3·1, i.e., at 150 μ*M* and 50 μ*M*, respectively (*see* **Note 6**). As a model system, we use PCR1 and PCR2 to amplify a 185-bp fragment from the hepatitis B virus (HBV) genome.
2. Add target DNA For the production of vector-free probe from plasmid DNA, the amount of plasmid DNA should be kept low, at 0.2 fmol (approx 1 ng of an 8-kb plasmid) (*see* **Note 7**)

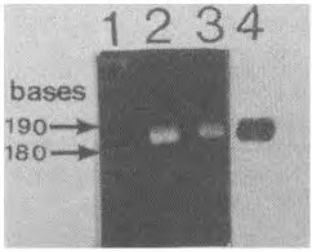

Fig. 1. Synthesis of an 185 bp biotinylated HBV probe using PCR labeling. Lane 1, pBR322 DNA digested with *Msp*I (marker); lane 2, nonbiotinylated PCR product; lane 3, biotinylated PCR product; lane 4, Southern blot of biotinylated PCR product (alkaline denaturing gel). Reprinted with permission from **ref. *3***.

3. Carry out thermal cycling. For PCR1 and PCR2, the thermal profile is: 94°C for 10 min, followed by 25 cycles of 94°C for 2 min, 55°C for 2 min, and 72°C for 3 min.
4. Remove as much mineral oil as possible.
5. Add 1 µL of glycogen (20 mg/mL) (Boehringer Mannheim, East Sussex, UK), 10 µL of 2.5 *M* sodium acetate, pH 5.2, and 220 µL of ethanol. Leave the mixture at –20°C overnight or –70°C for 1 h.
6. Spin down the precipitated labeled PCR product by centrifugation at 11,600g on a Micro Centaur (MSE, Sussex, UK) for 15 min. Discard the supernatant and vacuum desiccate.
7. Dissolve the precipitated probe in 100 µL of 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
8. Electrophorese 5 µL on an agarose gel. The biotinylated PCR product runs slightly slower compared with the corresponding nonbiotinylated PCR product (**Fig. 1**).
9. The efficiency of the labeling may be checked by running 1 µL of the PCR product on an alkaline denaturing agarose gel, Southern blotting to a nitrocellulose filter (BA 85, Schleicher and Schuell, Keene, NH), and detecting the biotin label by using a streptavidin/alkaline phosphatase system ***(5)*** (*see* **Subheading 3.2.**) (**Fig. 1**).
10. Store labeled probe at –20°C or –70°C in aliquots.

3.2. Filter Hybridization Using PCR-Labeled Biotinylated Probe

As a guideline, in the example using the 185-bp biotinylated PCR probe, 50 ng of the probe in 2 mL of hybridization mix is used for every 50 cm^2 of nitrocellulose filter.

1. Following standard hybridization procedures ***(5)***, wash the filters for 5 min using 2X Tris buffered saline (TBS), 0.1% (w/v) sodium dodecyl sulfate (SDS) at 22°C. Repeat the washing step twice more, making a total of three times.
2. Wash three times, each for 5 min with 0.5X TBS, 0.1% SDS at 22°C.
3. Wash three times, each for 15 min with 0.5X TBS, 0.1% SDS at 60°C. Prior heating of the washing solution to 60°C is essential.
4. Block filters with blocking buffer for 60–90 min.

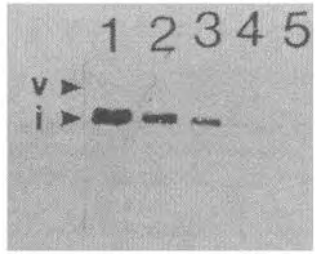

Fig. 2. Sensitivity and insert-specificity of the 185 bp PCR-generated biotinylated probe using serial dilutions of pHBV130 restricted with *Xho*I to release insert. v and i mark the positions of vector and insert, respectively. The amounts of insert in the lanes are: lane 1, 1 ng; lane 2, 100 pg; lane 3, 10 pg; lane 4, 1 pg; lane 5, 0.1 pg. Reprinted with permission from **ref. *3***.

5. Dilute streptavidin to 2 μg/mL in incubation buffer AP7.5 , 0.05% Tween-20.
6. Incubate filters with diluted streptavidin for 10 min with gentle rocking. As a guideline, use 3–4 mL of diluted streptavidin per 100 cm^2 filter area.
7. Wash filters three times, each with blocking buffer for 5 min.
8. Make up biotinylated alkaline phosphatase to 1 μg/mL in incubation buffer.
9. Incubate filters with biotinylated alkaline phosphatase for 10 min with gentle rocking.
10. Repeat the wash three times, each with blocking buffer for 3 min.
11. Wash filters twice, each with substrate buffer AP 9.0 for 3 min.
12. Make up nitro blue tetrazolium (NBT) (Sigma) and 5-bromo-4-chloro-3-indoly phosphate (BCIP) (Sigma) stock solutions by dissolving 1 mg NBT in 40 μL 70% dimethylformamide and 2 μg BCIP in 40 μL 100% dimethylformamide.
13. Make up substrate solution by adding 36 μL and 40 μL stock solutions of NBT and BCIP, respectively, to 12 μL of substrate buffer at 22°C.
14. Incubate filters with substrate solution for up to 20 h in plastic Petri dishes or sealed plastic hybridization bags in the dark. Monitor the color development periodically. Under optimal conditions, 1 pg of target DNA could be detected by Southern blotting using PCR-labeled biotinylated probe (**Fig. 2**).
15. Following color development, wash filters extensively with water.

3.3. Incorporation of Digoxigenin-11-dUTP

1. Set up the PCR reagent mix with 200 μ*M* each of dATP, dGTP, and dCTP. Use dTTP and digoxigenin-11-dUTP at a ratio of 3:1, i.e., at 60 μ*M* and 20 μ*M*, respectively (*see* **Note 6**).
2. Add target DNA. For the production of vector-free probe from plasmid DNA, the amount of plasmid should be kept low at 0.2 fmol (approx 1 ng of an 8-kb plasmid).
3. Carry out thermal cycling. The thermal profile is: 94°C for 10 min, followed by 30 cycles consisting of 94°C for 1.5 min, 60°C for 1.5 min, and 72°C for 3 min. This profile is found to work well with the primer combinations listed in **Table 2**.
4. Remove as much mineral oil as is possible.

Table 2
Product Size and Genomic Location of Primer Combinations

Primer combination	PCR product size (bp)	Location in HBV genome
PCR1/PCR2	185	Core-polymerase region
BB1666/BB1667	233	Surface antigen gene
BB1660/BB1661	401	Surface antigen gene
BB1666/BB1671	749	Surface-polymerase region
BB1660/BB1671	777	Surface-polymerase region

5. Electrophorese 10 µL of the PCR product on an agarose gel. The digoxigenin-labeled PCR product should exhibit decreased electrophoretic mobility compared with a nondigoxigenin-labeled PCR product.
6. The efficiency of labeling may be checked by electrophoresing 1 µL of the PCR product on an alkaline denaturing agarose gel, Southern blotting to a nitrocellulose filter (BA 85, Schleicher and Schuell). Detection of the digoxigenin label is detailed below.
7. Store probe at either –20°C or –70°C in aliquots.

3.4. Production of Single-Stranded Digoxigenin-Labeled Probe

For the production of single-stranded probes, two rounds of PCR are performed: the first round without the label and a second round of asymmetric PCR for label incorporation (*see* **Notes 1**, **2**, and **10**).

1. Set up the first round PCR using 200 µ*M* dTTP without dig-11-dUTP.
2. Following the first round, centrifuge the product through a Centricon-30 membrane (Anachem, Luton, UK) to remove the free primers.
3. Set up a second round of linear amplification using only one primer (either one of the primers used in the first round or an internal one), 20 µ*M* dig-11-dUTP and 60 µ*M* TTP. Sixty nanograms of the first round product is used as the template for the second round. Thirty cycles (94°C for 1.5 min, 60°C for 1 5 min, and 72°C for 3 min) are performed.

3.5. Use of Digoxigenin-Labeled Probe in Filter Hybridization

As a guideline, digoxigenin-labeled PCR probes are used at a concentration of 50 ng/µL of hybridization mix.

1. Following standard hybridization procedures *(6)*, wash filters twice, each for 5 min at 22°C with 2X SSC, 0.1% (w/v) SDS.
2. Wash twice, each for 15 min at 65°C with 0.1X SSC, 0.1% SDS.
3. Block filters for 1 h at 22°C with 1X AP7.5, 0.5% Tween-20.
4. Dilute antidigoxigenin alkaline phosphatase Fab fragment (Boehringer Mannheim) to 150 mU/mL (1: 5000) with 1X AP7.5, 0.05% Tween-20, 2% bovine albumin (Sigma).
5. Incubate filters with antidigoxigenin for 30 min at 22°C.

6. Wash twice, each for 5 min with 1X AP7.5, 0 05% Tween-20.
7. Wash twice, each for 5 min with 1X AP7.5.
8. Wash twice, each for 5 min with 1X AP9.0
9. Make up nitro blue tetrazolium (NBT) (Sigma) and 5-bromo-4-chloro-3-indoly phosphate (BCIP) (Sigma) stock solutions by dissolving 1 mg NBT in 40 µL 70% dimethylformamide and 2 mg BCIP in 40 µL 100% dimethylformamide
10. Develop filters with substrate solution (36 µL NBT solution and 40 µL BCIP solution / 12 mL of 1X AP9 0). Under optimal conditions, between 1 and 10 pg of target DNA could be detected on Southern blotting using a PCR-generated digoxigenin probe.

3.6. In Situ *Hybridization Using PCR-Generated Digoxigenin Probes*

1. Put 5-µm sections onto 1% silane treated slides using sterile distilled water and dry overnight at 22°C
2. Heat section for 10 min at 80°C. Then dewax in Citroclear (HD Supplies, Aylesbury, UK) three times, each for 5 min, followed by industrial methylated spirits three times, each for 5 min. Hydrate in sterile distilled water.
3. Protease digest to access nucleic acid target This step is crucial–too much digestion results in loss of morphology and nucleic acid (especially mRNA), too little results in reduced sensitivity and higher background. Incubate slide in Protease 8 (Sigma) in PBS at 37°C with gentle shaking. Concentration and time depend on tissue type and fixation Typically, we use a concentration of 50 mg/mL for 20 min at 22°C for DNA target and a concentration of 5 mg/mL for 20 min at 22°C for RNA.
4 Rinse slide in TBS
5 For DNA target, denature slide/section in sterile distilled water at 95°C for 20 min. For RNA, denature slide/section in 70% formamide in 2X SET at 37°C for 20 min.
6. Rinse in ice cold water 3 min, air dry.
7 Prepare mix: 100 µL sterile distilled water, 100 µL 0.1% SDS, 100 µL 10 mg/mL polyvinyl pyrrolidone, 400 µL 50% dextran sulphate in 10X SET, 1000 µL deionized formamide. Mix and filter through 0.2 µm filter. Divide into 170 mL aliquots and store at –20°C. Before use add 10 µL 10 mg/mL salmon sperm DNA and 20 µL probe at 10 mg/mL.
8. Denature probe/mix at 95°C for 20 min. As a guideline, we use PCR-generated digoxigenin-labeled probes at a concentration of 10 ng/section for *in situ* hybridization
9. Apply 7 to 16 µL of denatured probe/mix to coverslip and invert section onto probe/mix and seal with silicone grease.
10. For DNA, denature probe/section again by placing on a hot plate at 95°C for 10 min.
11. Hybridize at 37°C for 2–24 h in a moist Petri dish. Time depends on amount of target and concentration of probe (higher requires less time)
12 Remove coverslips in TBS/0.5% Triton X100 and rinse three times, each for 5 min at 37°C in same. Then rinse three times, each for 5 min in 0 5X TBS at 65°C.
13 Block with 15% dried milk in AP7.5/0.5% Triton X100 for 20 min at 37°C.

14. Rinse and apply alkaline phosphatase conjugated antidigoxigenin (Boehringer Mannheim) 1 to 750 in 2% BSA/AP7.5, 0.5% Triton X100 for 30 min at 37°C
15. Rinse in AP7.5/0.5% Triton X100 three times, each for 5 min at 22°C, then in AP9.0 three times, each for 5 min at 22°C.
16. Develop in NBT/BCIP as for filters (typically overnight).

4. Notes

1. The specificity of PCR-generated single strand probes can be confirmed by demonstrating that hybridization only occurs to mRNA with antisense strand probes, but not with sense strand probes.
2. When used for *in situ* hybridization, single strand probes are found to be more sensitive than double strand ones.
3. Longer probes are generally found to be more sensitive than shorter ones When two or more small probes that have overlapping sequences are used concurrently, an additive effect in terms of sensitivity is seen.
4. The recent development of long range PCR technology *(7)* has provided the possibility of producing longer probes than previously achievable.
5. It is also possible to generate labeled probes by using primers with 5' end labels, e.g., biotin. However, this approach has lower sensitivity than the approach outlined in this chapter because only a single label molecule is available subsequently for detection using the labeled-primer approach. However, the labeled-primer approach does have the advantage that, compared with internally labeled probes, steric hindrance is less likely to be a problem. It has been demonstrated that internally labeled biotinylated probes have reduced T_m *(8)*. This point would need to be taken into consideration when using PCR-labeled probes in hybridization experiments.
6. The ratio of the labeled-deoxynucleotide triphosphate and dTTP should be carefully controlled. In our laboratory, we use 150 μM dTTP to 50 μM biotin-11-dUTP and 60 μM dTTP to 20 μM digoxigenin-11-dUTP for the biotin and digoxigenin-labeled system, respectively.
7. When the source of probe material is plasmid DNA and it is necessary to produce vector-free probe, it is important to use a relatively small amount of plasmid DNA (approx 0.2 fmol or 1 ng of an 8 kb plasmid). The use of larger amounts of starting plasmid DNA will result in a significant amount of labeled vector sequences.
8. For probe sequences that have been cloned into expression vectors, it is possible to use universal primers to the RNA polymerase promoter sites as amplification primers. Primer sequences to the T_3, T_7, and SP6 promoter sites are listed in **Table 1**. For example, the T_3 and SP6 primers can be used as a pair in PCR for insert flanked by the T_3 promoter on one side and the SP6 promoter on the other.
9. PCR can also be used to produce labeled probes from genomic DNA. Due to the sequence complexity of DNA, we suggest that an initial round of PCR using conventional deoxynucleotide triphosphates, without the label, is first performed. The PCR product is then gel-purified and then a second round of PCR is carried out by including the labeled deoxynucleotide triphosphate to label the product. High background may result if this two-step approach is not used.

10 Single-strand PCR-labeled probes are at least twice as sensitive as their double strand counterparts of the same size, possibly due to the absence of reannealing. Single strand probes also have the advantage of providing strand specificity We find that heating prior to hybridization seems to improve the sensitivity of experiments carried out using single strand probes, probably as a result of the denaturation of the secondary structure of these single strand molecules.
11. Incorporation of biotin labels during PCR has also been used as a means of quantifying PCR products *(9)*

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11

PCR for the Detection of Minority DNA Populations

Y. M. Dennis Lo

1. Introduction

Although the polymerase chain reaction (PCR) is generally believed to be capable of detecting a single target DNA molecule, it is important to note that there are essentially two different types of "low copy number PCR." The first type involves the amplification of an isolated DNA target, such as single sperm analysis *(1)* and preimplantation embryo diagnosis *(2)* (*see* Chapter 20). In these examples the PCR target is surrounded by PCR buffer and there is no background DNA to interfere with the reaction. In the second category, the DNA target is surrounded by an excess of background DNA molecules. This is a common situation in many clinical scenarios from many different research and diagnostic disciplines, such as the molecular monitoring following bone marrow transplantation (*see* Chapter 15) and noninvasive prenatal diagnosis (*see* Chapters 21 and 22). This chapter reviews the theoretical and practical considerations of minority DNA population detection by PCR.

2. Theoretical Considerations of Minority DNA Populations Detection by PCR

2.1. Sensitivity Considerations

Sensitivity of PCR is probably its most important attribute with regard to detecting a minority nucleic acid population. The presence of background DNA frequently gives rise to nonspecific amplification products that reduce the overall sensitivity of the assay. Thus, in order to obtain the maximum sensitivity, modification of the basic PCR methodology is required. Two such modifications are commonly used.

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2.1.1. Nested PCR

In the nested PCR strategy, PCR product from a first round PCR is reamplified using a second pair of primers "internal" to the original primer pair in a second series of amplification cycles. The effect of this second round PCR is to select out the specific PCR product from any nonspecific first round products, using internal sequence information that is only present on the specific PCR product. Using this strategy, the sensitivity is enhanced from 100 to 1000 times, to an extent that even a single copy target can be detected in a complex background of 300,000 cells or more. This specificity and sensitivity enhancement is also observed when the second round PCR is carried out using one internal primer and one of the original external primers—a strategy termed "heminesting." Recently a number of strategies have also been described for carrying out nested PCR in a single tube *(3–5)*.

2.1.2. Hot-Start PCR

In hot-start PCR, one or more of the reagents necessary for PCR is withheld either physically or functionally from the reaction mix until the temperature of the reaction tube is above 50°C. This approach has been found to improve the specificity and sensitivity of the PCR *(6,7)*. This improvement has been hypothesized to be due to the fact that the *Taq* polymerase has significant activity at room temperature. As the target DNA may contain single strand regions created during the DNA extraction process, primers may anneal nonspecifically to these regions at the low ambient temperature during the PCR set-up phase *(7)*. These annealed primers are then extended by the *Taq* polymerase, thus forming nonspecific products that will compete with the specific target during subsequent amplification cycles.

The production of these nonspecific products is avoided if the full complement of PCR reagents is only made available or active at an elevated temperature, e.g., above 50°C. Hot start is a generic term describing diverse strategies aimed at achieving this effect. Early approach to hot start involves the manual addition of the withheld reagent(s) when the PCR tubes have reached a certain temperature, a procedure that is inconvenient, time-consuming, and prone to contamination. Hot start PCR has been simplified using a wax barrier that physically separates the various reaction components at room temperature and melts down when the reaction temperature has reached a certain temperature, to allow the reagents to mix with each other *(7)*. However, Ampliwax technology is not the only way to achieve automated hot start. Recently, a new DNA polymerase, called Ampli*Taq* Gold, which is inactive at room temperature but activated by heat, has been introduced and is likely to further simplify the hot-start process *(8)*. With hot-start PCR, single molecule sensitivity in a complex environment is possible without nesting

and without radioactive probing *(7)*. Thus, hot start PCR greatly simplifies high sensitivity PCR procedures, making the method more suitable for clinical use.

2.1.3. Detection Systems

The most common method for detecting PCR products is agarose gel electrophoresis and ethidium bromide staining. Although this method is adequate and convenient, there are much more sensitive and specific detection systems available, which are most useful when extremely rare target sequences are detected. Southern blotting and probing of the PCR products is one widely used method to achieve sensitivity and specificity enhancement. The use of fluorescence-labeled primers and the use of a computerized fluorescence-based scanner is a more convenient and probably equally sensitive method to achieve the same purpose (*see* Chapter 20).

2.2. The Sampling Problem

With decreasing numbers of target DNA molecules, the probability of having a target molecule in a test sample decreases and is described by the Poisson distribution:

$$P(n) = (e^{-m} m^n)/n! \qquad (1)$$

where $P(n)$ is the probability of having n molecules per test aliquot, m is the mean number of molecules in the sample, and e is a constant (2.718 .).

For example, when there is an average of two target molecules per test aliquot, then the probability of having no target molecule in a particular test is 0.14; when the number of target molecules decreases to an average of one molecule per test aliquot, the probability increases to 0.37. These considerations highlight the need for performing multiple tests for extremely rare targets. Sampling error is likely also to contribute in part to the many discrepancies in the literature regarding the sensitivity of particular PCR assays.

2.3. Effect of the Amount of DNA Used in an Assay

The amount of DNA used in a PCR assay limits the maximum sensitivity of a particular assay. For example, 1 μg of DNA is derived from approximately 150,000 cells, with approx 6.6 pg per cell. Thus, when detecting a sequence that is present once per target cell, e.g., a single copy gene on the Y chromosome, the maximum sensitivity of the assay is 1 in 150,000. Increasing the amount of DNA used in an assay can theoretically increase the sampling base and thus the potential sensitivity of the assay. However, this issue has to be balanced against the detrimental effect of increasing the amount of template DNA, such as the increase in nonspecific amplification and the presence of inhibitors in DNA *(9)*.

2.4. Choice of Targets

In certain situations, there exists more than one target sequence that may be used for PCR amplification. Notable examples include the detection Y-chromosomal sequence of a male fetus from maternal blood *(10,11)*or the detection of residual host cells from a male bone marrow transplant recipient who has been transplanted with female marrow *(12)*. In these examples, consideration has to be given to the characteristics of the various Y targets such as their copy number and their relative Y-specificity. High copy number targets such as the DYZ1 (a repetitive Y sequence that repeats from 800–5000 copies per male cell) *(10)* have the advantage that they are usually highly sensitive and allows one to detect "a fraction" of a cell. The disadvantage of such high copy number targets is their possible lack of Y-specificity. For example, it has been suggested that the DYZ1 locus exhibits a degree of autosomal cross-reactivity that may explain the proneness of DYZ1 PCR to false-positivity *(13)*. Another disadvantage of high copy number targets is that they further accentuate the problems of exogenous contamination.

A second example in which more than one type of target may be used is found in studies investigating the detection of circulating cancer cells in peripheral blood of patients (*see* Chapters 18 and 19). In these studies, both tumor-specific DNA or RNA sequences may be used. RNA-based studies have the advantage that the existence of multiple copies of mRNA per tumor cell allows the development of extremely sensitive assays. This advantage, however, has to be balanced against the greater lability of RNA targets and the greater complexity of RT-PCR over conventional PCR.

For nucleic acid targets that are present in multiple copies per cell it may be argued that extracted nucleic acids may constitute a better source of starting material than whole cells because the multiple target molecules are released into the solution during the extraction procedure and thus maximizing the chance for having a target molecule in the test aliquot used for PCR. On the contrary, if whole cells are sampled directly for PCR, the multiple target nucleic acid molecules contained inside a cell will behave as a single unit and thus be more likely to be subjected to sampling error.

2.5. Target Cell Enrichment

Target enrichment aims at concentrating target materials such that the reliability of a subsequent diagnostic step may be increased. Even though this has been achieved in some instances, e.g., fetal cells in maternal blood (*see* Chapters 21 and 22) and circulating cancer cells (Chapter 19), the general lack of specific markers for enrichment and the fact that many enrichment methods (e.g., the fluorescence-activated cell sorter) are relatively labor-intensive, mean that in many instances PCR has to be used on samples not previously enriched

for the target cell types. One ingenious method of target cell enrichment is the use of micromanipulation to pick up individual target cells and subject each or pools of these cells to amplification. Notable success has been achieved in the field of noninvasive prenatal diagnosis *(14–16)*. Micromanipulation has the important effect of reducing the problems caused by the majority DNA background (*see* **Subheading 2.6.**).

2.6. Effect of Majority DNA Background

The general effect of the DNA background in reducing sensitivity has been mentioned in **Subheading 2.1.** This section focuses on the problems imposed by the DNA background when using PCR to detect a minority target that shares homology with the background DNA population. This constitutes a challenging and clinically important problem because in many diagnostic settings the majority and minority populations differ by only a small number of nucleotides, e.g., a mutant *ras* allele amongst a majority of cells possessing the wild-type *ras* gene. Theoretically, there are two ways to approach this problem. The first involves the use of an amplification system that will amplify both the majority and minority sequences that are then distinguished from one other by a subsequent analytical step. The main problem with this strategy is that the PCR plateau of 3–5 pmol *(17)* is then shared between the majority and minority PCR products. As the background molecules at the start of the PCR is in great excess when compared with that of the target minority, most of the resulting PCR products originate from the background. In other words, the amplification factor for the target sequence is limited. The best studied examples of systems in which the majority and minority populations are coamplified may be found in the literature on post bone marrow transplantation chimaerism *(18)* in which the typical sensitivity of these assays ranges from 0.1% to 1%.

The second type of approach involves the design of amplification systems that will specifically amplify the minority target sequence, while remaining refractory to the related, but not identical background sequence. The amplification refractory mutation system (ARMS) *(6,19,20)* is a type of PCR that is capable of achieving this aim. ARMS allows the PCR to be discriminatory down to a single nucleotide level. The principle and application of ARMS has been discussed in detail in Chapter 7.

2.7. Contamination

The main strength of PCR, namely its sensitivity, is also its chief weakness as it is extremely prone to false-positive results due to contamination *(21,22)*. This disadvantage is especially obvious in applications requiring the highest sensitivity, such as in the detection of minority DNA populations. Guidelines aimed at reducing the risk of PCR contamination have been discussed in Chapter 2.

3. Conclusions

The detection of minority nucleic acid populations using the PCR is a rapidly developing field. The number of uses is likely to increase in the near future as investigators from diverse fields begin to realize the power of the technique. Further improvement in amplification methodology will be expected to further improve the robustness and clinical applicability of this promising technology.

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12

In Situ Amplification

John O'Leary

1. Introduction

A major limitation of solution phase polymerase chain reaction (PCR) is the inability to visualize and localize amplified product within cellular and tissue specimens. *In situ* hybridization (ISH) does permit localization of specific nucleic acid sequences at the individual cell level. In conventional nonisotopic *in situ* (IS) detection systems, most protocols do not detect single copy genes, except for those incorporating elaborate sandwich detection techniques as described by Herrington et al. *(1)*.

Before amplification can be carried out in solution phase PCR (SPPCR), nucleic acid extraction is first required, which necessitates cellular destruction. Subsequent correlation of results with histologic features is consequently not possible. Recently, a number of studies have described the use of PCR with ISH *(2–6)*. However, problems are encountered with the technique, with reaction failure or false positive signals commonly seen. The repertoire of IS amplification techniques has now been extended to include primed IS labeling (PRINS) and cycling PRINS *(7)*.

1.1. Definitions and Terminology

Five essentially similar techniques are described.

1. DNA IS-PCR: Refers to PCR amplification of cellular DNA sequences in tissue specimens using either a labeled probe or labeled oligonucleotide (i.e., deoxyuride triphosphate [dUTP]) within the PCR reaction mix. The labeled product is then detected using standard detection techniques as for conventional ISH or immunocytochemistry.
2. PCR-ISH: Refers to PCR amplification of cellular DNA sequences in tissue specimens followed by ISH detection of the amplified product using a labeled

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internal or genomic probe The labels used can either be isotopic (^{32}P, ^{35}S) or nonisotopic (e g., biotin, digoxigenin, fluorescein). To date, most studies have utilized nonisotopic labels.

3 IS reverse transcriptase (RT)-PCR-(IS RT-PCR): Refers to the amplification of mRNA sequences in cells and tissues specimens by first creating a copy DNA template (cDNA) using RT and then amplifying the newly created DNA template as for IS-PCR

4 RT PCR-ISH: Refers to amplification of RNA sequences in cells and tissues specimens by creating a cDNA template using RT, amplifying the newly created DNA template, and then probing this DNA with an internal oligonucleotide probe.

5. PRINS and cycling PRINS· Amplification of specific genetic sequences in metaphase chromosome spreads or interphase nucleic, using one primer to generate single-stranded PCR product. If many rounds of amplification are utilized then the technique is called cycling PRINS

1.2. Principles of the Techniques

IS-PCR and PCR-ISH techniques represent the coming together of PCR and ISH, allowing the amplification of specific nucleic acid sequences inside cells. Initially, cells or tissues are fixed with a suitable fixative (usually a formaldehyde fixative, e.g., neutral buffered formaldehyde [NBF]) and are then permeabilized using proteolytic enzymes in order to permit access of PCR reagents into the cells and to the target nucleic acid. IS amplification can be performed either in intact cells in Eppendorf tubes (as in SPPCR) or on cytocentrifuge preparations or tissue sections on glass microscope slides.

Protocols for IS amplification differ greatly between research groups and depend on the target to be amplified and the particular tissue under investigation. Haase et al. *(2)* described IS-PCR in fixed single cells, suspended in PCR reaction buffer. After amplification, cells were cytocentrifuged onto glass slides and the amplified product detected using ISH.

Other early approaches used pieces of glass slides in standard Eppendorf tubes, incubated directly in PCR reaction buffer with attached cells from cytocentrifuge preparations *(8)*. More recently, techniques using tissues and cells attached to microscope slides have been described with amplification carried out either on heating blocks or in cycling ovens *(3–5,9)*. Initially, investigators used standard multiwell PCR blocks. To create an amplification chamber, aluminium foil boats were used onto which the slide containing the tissue section was placed. However, optimization of thermal conduction was never successfully achieved using standard thermal cycling blocks with "thermal lag," i.e., differences in temperature between the block face, the glass slide, and the PCR reaction mix at each temperature step of the reaction cycle commonly encountered *(5)*. The newer IS amplification machines, which offer built-in slide temperature calibration curves, correct for this thermal lag phenomenon.

1.3. Tissue Fixation and Preparation

1.3.1. Cell and Tissue Fixation

The cytoskeleton of the cell is first made rigid in order to create a microenvironment within the cell, which facilitates entry of PCR reagents and minimizes leakage of PCR product. Successful DNA amplification has been achieved with tissues fixed in 1–4% paraformaldehyde, NBF, and 10% formalin for 12–24 hours *(4,5)*. Occasionally, ethanol and acetic acid fixed tissues may be amplified, but these invariably give variable results. Unfortunately, fixation of cells with formaldehyde fixatives provides a number of drawbacks. Formaldehyde is not easily removed from tissues, even after tissue processing. Aldehyde groups can react with nucleic acid template to form DNA–DNA and DNA–histone protein crosslinks *(10)*. Formaldehyde fixation also allows "nicks" to occur in the DNA template, some of which are nonblunt ended and can subsequently act as potential priming sites for extension by *Thermus aquaticus* (*Taq*) DNA polymerase; the process even occurs at room temperature. This can lead to spurious results, particularly with IS-PCR, when direct incorporation of labeled nucleotide is used.

1.3.2. Cell and Tissue Adhesion

Attachment of cells to glass slides must be carried out in order to prevent detachment during the repeated cycles of heating and cooling during amplification. Slides pretreated with coating agents ensure maximal section adhesion. The most commonly used coating agents are aminopropyltriethoxysilane (APES), Denhardt's solution, and Elmer's glue.

1.3.3. Exposure of Nucleic Acid Template

Cell permeabilization is carried out in order to facilitate entry of reagents into the cell. This can be achieved by several methods, including mild protease treatment or mild acid hydrolysis (0.01 *N*–0.1 *N* HCl) In SPPCR, extensive proteolytic digestion (occasionally up to 48 h) is employed to overcome the problem of DNA–DNA and DNA–histone protein crosslinking. However, this cannot be performed with IS-PCR techniques, as extensive proteolysis destroys cellular morphology. Maximal digestion time is approx 20–25 min. Longer digestion times compromise cellular architecture. Incomplete dissociation of histone protein–DNA crosslinks occurs in IS-PCR techniques. Acid hydrolysis probably acts by driving such crosslinks to complete dissociation.

1.4. Amplification: Reagents and Conditions

Setting up the amplification step, as for SPPCR, involves the careful choice of target sequence (taking into account its specific melting temperature, T_m),

the choice of primers (again taking into account their T_m, their ability to form primer–dimers, and uniqueness), and the careful optimization of the PCR cycling parameters. Initial denaturation of DNA can be achieved at the beginning of thermal cycling or separately during permeabilization or alternatively, following the fixation process. Denaturation can be achieved using heat, heat/formamide, or alkaline denaturation *(11,12)*. Most investigators advocate the use of "hot-start" PCR to reduce mispriming and primer oligomerization. Nuovo suggested the use of single strand binding protein (SSB) derived from *Escherichia coli*, which is involved in DNA replication and repair to prevent primer mispriming and oligomerization *(3)*. The precise mode of action is unknown.

The volume of PCR reagents varies, depending on the size of the cell preparation/tissue specimen that one wishes to amplify. In general, 15–75 µL of PCR reagents are used for most IS-PCR techniques that use glass mounted material. Small variations in the volume of these reagents over the slide can give rise to localized PCR failure contributing to "patchy" results. When using the Gene Amp IS-PCR 1000 system, 50 µL is advised. The optimal number of cycles is dependent on the particular assay. The lower the number of cycles, the better, since product diffusion problems are less likely to be encountered and cellular morphology maintained. Most protocols use 25–30 cycles of amplification; exceptionally 50 cycles. Some groups have used two rounds of 25–30 cycles with the addition of new PCR mixture and *Taq* DNA polymerase or nested PCR with two rounds of 30 cycle amplification. Primer selection has evolved around two basic strategies: single primer pairs *(9,15)* or multiple primer pairs with or without complementary tails. The multiple primer pair approach was designed to generate longer and/or overlapping product, with less potential to diffuse from the site of manufacture. In general, single primer pairs suffice if the "hot-start PCR" modification is adopted.

1.5. Postamplification Washing and Fixation

Most IS amplification protocols include a postamplification washing step, mainly in order to remove diffused extracellular product and reduces the chances of generating false positive results. Postfixation with 4% paraformaldehyde and/or ethanol is also used to maintain localization of product.

1.6. ISH for Detection/Confirmation of Amplified Product

Recombinant DNA technology now provides the opportunity to use DNA or RNA probes to any desired sequence. Furthermore, one can choose between single- and double-stranded probes. The type of probe to be used depends greatly on the target sequence. Probes to DNA viruses are constructed as recombinant plasmids or cosmids, with whole viral DNA or restriction enzyme

fragments of specific viral sequences. Many viral and human probes are now commercially available, and may be obtained from the originating laboratory or from the American Type Culture Collection. Double-stranded probes, if randomly sheared, form networks on the cytological hybrid and so increase the hybridization signal. However, double-stranded probes can also anneal in solution and thus reduce the concentration of probe available for reaction with the cytological preparation. Recently, human and viral probes have been constructed synthetically using oligonucleotide synthesizers (oligonucleotide probes). These oligonucleotides containing complimentary sequences to DNA or RNA viruses or human sequences can be easily labeled and then used as probes. Their use in conjunction with nonisotopic detection systems has been limited because the short sequences of these probes allow less room for adequate labeling. To use such oligonucleotides successfully in nonisotopic methods, it is necessary to use cocktails containing up to four different probe sequences.

The general use of ISH is hindered by problems associated with probe preparations. Initially probes were prepared using crude isolated nucleic acids. In addition, only cumbersome, expensive, and dangerous radioactive labels could be used. These difficulties have been largely overcome by recombinant DNA technology. The development of a biotinylated nucleotide analog, biotin 11-dUTP (Enzo Diagnostics, New York, NY) and the development of DNA labeling by nick translation greatly facilitated the use of nonisotopic ISH (NISH). The use of biotinylated viral probes was initially described using immunocytochemical detection, but the development of a technique using a biotin/streptavidin/alkaline phosphatase sandwich detection system proved to be more sensitive. Subsequently, multiple sandwich immunohistochemical techniques have recently become available. Problems exist when using biotin, as many tissues, including liver, small bowel, and endometrium, have high levels of endogenous biotin, which then interferes with the detection of the biotinylated hybridization products. Alternative molecules used include aminoacetylfluorene- and mercury-labeled probes that allow the detection of nucleic acid sequences in fresh isolates or cultured cells by fluorescent methods. Generally, fluorescence detection systems are not readily applicable to archival paraffin wax embedded sections because of endogenous autofluorescence. However, digoxigenin, a derivative of the cardiac glycoside digoxin, conjugated to deoxyuridine triphosphate is commonly exploited. Most DNA probes (especially viral) contain long double-stranded sequences and consequently nick translation is the most general and effective method for labeling probes with either biotin or digoxigenin. Nick translation has the advantage of producing large quantities of labeled probe that can be stored readily at –20°C for up to 2 yr without any significant deterioration, and gives

probes of optimum size (i.e., 300–800 bp). Labeling an entire recombinant plasmid, so the probe can be used with "built-in carrier DNA" enhances signal strength. Random primer labeling methods yield low levels of product (70 ng) and requires predigestion of the plasmid giving excised fragments that include the viral sequence. Among the biotin deoxynucleotides available, biotin 11-dUTP allows optimum detection sensitivity. Separation by spun column purification removes unincorporated nucleotides from labeled probes and therefore reduces high levels of background staining.

1.7. Posthybridization Washing

Posthybridization washes are performed most efficiently with agitation. This can be accomplished simply by using an orbital shaker or inverting a Coplin jar lid into the bottom of a glass staining dish and placing a magnetic follower ("magnetic flea") in the upturned lid. Fill the dish with wash buffer and immerse the staining rack into the buffer so that it rests on the edge of the Coplin jar lid. The buffer can now be stirred vigorously on a magnetic stirrer without interference to the magnet's motion.

1.8. Visualization of PCR Product

Nonisotopic labels provide similar degrees of detection sensitivity to isotopic labels. For ISH after amplification, maximum specificity should only be achieved by probes that recognize sequences internal to the amplified product. However, genomic probes can also be used, which are not restricted to the primer-free sequences and appear to provide similar results.

Postamplification immunohistochemistry has been described by Nuovo *(3)*, but most investigators find this difficult to perform, because many epitopes do not appear to withstand the repetitive changes of thermal cycling.

1.9. Reaction, Tissue, and Detection Controls

Judicious use of controls is advised. All reaction setups require reference control genes, known negative samples for the target sequence together with irrelevant primers, and irrelevant probes. Parallel SPPCR should also be performed for each IS-PCR assay. The following are the minimal controls required in the PCR-ISH:

1. Reference control gene, e.g., β-globin or pyruvate dehydrogenase (PDH) (**Fig. 1**).
2. DNase digestion.
3. RNase digestion
4. Target primers with irrelevant probe.
5. Irrelevant primers with target probe
6. Irrelevant primers with irrelevant probe.
7. Reference control gene primers with the target probe.

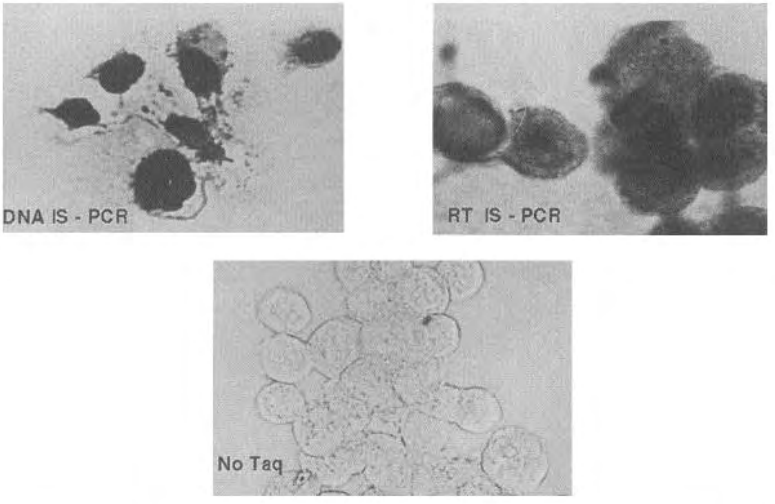

Fig. 1. PDH gene amplification in SiHa cells. Note the localization of DNA signals and RNA signals within the cells.

8. Target primer 1 only.
9. Target primer 2 only.
10. No *Taq* (**Fig. 1**).
11. No primers.
12. Omit the RT step in RT IS-PCR or RT PCR-ISH.
13. ISH controls for PCR-ISH and RT PCR-ISH.
14. Detection controls for immunocytochemical detection systems.

For IS-PCR, controls steps 1–3, 8–10, 11, together with a setup including one target primer and one irrelevant primer pair are used.

Reference control genes are important to assess the degree of amplification in the tissues section/cell preparation, the use of a single copy mammalian gene such as PDH further allows discrimination of the assay. For amplification of the DNA target, DNase digestion should abolish the signal. If a signal is still present after DNase digestion, then the source of the product is either RNA/cDNA or has arisen from spurious amplification. In IS-RT-PCR, RNase pretreatment destroys cellular RNA sequences and for DNA assays reduces the chance of false positives being generated from RNA templates.

Irrelevant primers with irrelevant probe should not generate a PCR signal of the specific target. The use of the reference control gene primer pair and the target probe directly assesses the degree of "stickiness" of the target probe sequence and the propensity to generate false positive signal. Using only one primer of the target sequence achieves an "asymmetric PCR," with accumulation of minimal product which is not easily detectable.

Excluding *Taq* DNA polymerase examines the contribution of primer–primer dimerization and primer oligomerization in generating false positive signals. Excluding primers examines the role of nonspecific elongation by *Taq* DNA polymerase of nicked DNA in tissue sections. This is an extremely important control for ISH-PCR. Using target primers with irrelevant probe is required to assess the specificity of the ISH component of PCR-ISH. Omitting the RT step for RT-IS PCR is important as only RNA should be amplified if proper DNase digestion has been carried out.

ISH controls and detection controls are mandatory as for routine ISH, in order to exclude false positive/negative results because of failure of the ISH step or aberrant staining of the tissues by the detection system.

1.10. Problems Associated with the Techniques

To date, no universally applicable technique is available. The type of starting material, the target, the fixation conditions of the tissues, and so on, all appear to influence the reaction. Overall, IS-PCR does not work well with paraffin wax embedded material, because of the problem of nonspecific incorporation of nucleotide sequences by *Taq* DNA polymerase. PCR-ISH protocols work, if the hot-start modification is used and or if multiple primer pairs are utilized. A major advantage of these techniques is their sensitivity, although efficiency is compromised with almost linear amplification achieved. The level of amplification in any particular PCR is difficult to assess. Widely contradictory figures are given, Nuovo *(3)* reported a 200–300-fold amplification, whereas Embretson et al. *(6)* estimated amplification of the order of 10–30-fold, depending on the number of cycles. My own observation would tend to support the latter's estimation.

The problem of nonspecific incorporation of nucleotides by *Taq* DNA polymerase into damaged DNA is DNA polymerase and cycle dependent. It occurs frequently in the absence of primers or even with hot-start modification. This makes the IS-PCR technique using directly incorporated nucleotides extremely unsatisfactory and generally we believe unsuitable for routine use. Gosden et al. *(7)* reported the use of strand break joining in IS-PCR amplification carried out on chromosomes to eliminate spurious incorporation. Di-deoxy blockage during pretreatment may also minimize spurious incorporation, but not always completely effective. Strand "super-denaturation," in which double-stranded DNA is denatured at high temperatures and maintained at a denatured state for a prolonged time period (5–10 min), appears to eliminate nonspecific incorporation by *Taq* DNA polymerase, but again this is variable in its effect. This "super-denaturation" routinely precedes conventional "hot-start" PCR at 70°C (O'Leary, 1997, submitted).

1.10.1. Specific Problems

1. Sequestration of reagents: To carry out successful amplification, increased concentrations (2–5 times) of reagents are essential. The explanation for using such high concentrations is that some or all the reagents are sequestered during the reaction. Sequestration of reagents can occur by several methods: reagents adhering to the glass slides, the coating reagents on the slides, or by direct intercalation by the fixative residues present in the tissue. Coating slides with 0.1–1% bovine serum albumin (BSA) allows decreased concentrations of reagents to be used, possibly blocking this sequestration *(17)*.
2. Diffusion of amplicons: Diffusion of product from the site of manufacture is encountered. The lower the cycle number used, the less likely diffusion will occur. Postfixation with paraformaldehyde and ethanol sometimes helps to maintain localization of the product. Other strategies include overlaying the tissue section with a thin layer of agarose or direct incorporation of biotin-substituted nucleotides (as with IS-PCR), to render the product more bulky and less likely to diffuse.
3. Patchy Amplification: Patchy amplification is always encountered, with on average 30–80% of cells showing amplification signals for the desired target sequence. Patchy digestion during cell permeabilization steps, lingering fixative–DNA and DNA–histone protein interactions all account for this phenomenon. In addition, processed tissues are cut by microtome blades during routine histologic preparations, and the tops of many cells are truncated with two immediate effects. First, truncated cells may not contain the amplified product, giving rise to a negative result, and second, the product is more likely to diffuse from a truncated cell.

1.11. Current Applications

Several groups have worked with IS-PCR techniques, and have identified single copy sequences in cells and low copy number DNA sequences in tissue sections. Many studies have focused on the detection of viral DNA sequences in defined systems, such as the human immunodeficiency virus (HIV), human papillomavirus (HPV) (**Fig. 2**), mouse mammary tumor virus (MMTV) provirus, cytomegalovirus (CMV), hepatitis B virus (HBV), Kaposi's sarcoma-associated herpes virus (KSHV). Some groups have examined endogenous human DNA sequences, including single copy human genes, chromosomal rearrangements and translocations *(9,13,15)*. The ability to amplify tumor-specific nucleic acid sequences as possible "clonal markers" of malignancy (e.g., T-cell receptor gene rearrangements, translocations, point mutations) has enormous potential for the future, particularly in diagnostic tumor pathology.

2. Materials

2.1. PCR-ISH

2.1.1. Equipment

Many types of equipment have been used for IS amplification techniques, including standard heating blocks, thermal cycling ovens, and specifically

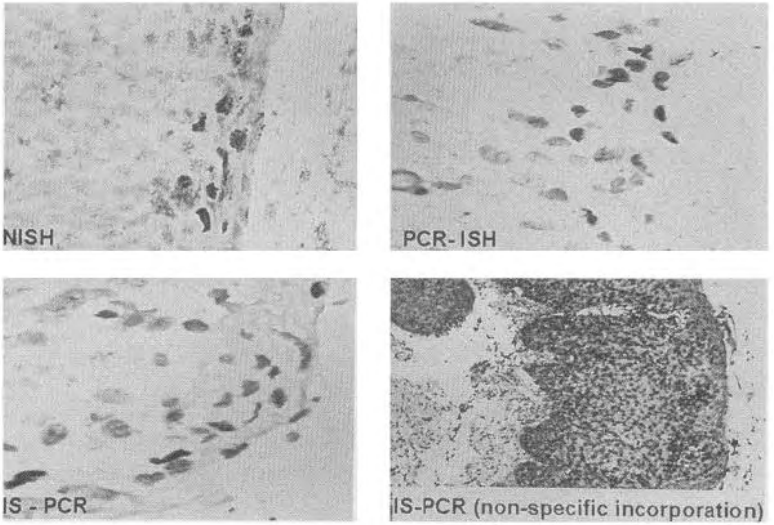

Fig. 2. NISH of HPV 16 in cervical intraepithelial neoplasia as compared to signals generated by PCR-ISH and IS-PCR and the nonspecific incorporation phenomena seen with IS-PCR.

designed IS-PCR thermocyclers, e.g., Gene Amp IS-PCR system 1000 (Perkin-Elmer) and the Omnislide/Omnigene machine (Hybaid, Teddington, UK). The latter specifically designed machines offer built-in slide calibration temperature curves, which optimize heat transfer kinetics from the thermocycling block to the glass slide and the tissue section.

2.1.2. Slide Preparation

1. Microscope slides: DNA/RNA sequences can be detected in cell smears, fresh, frozen, or paraffin-embedded sections by ISH or IS amplification techniques. The use of either single rectangle well slides (type PH 106 from Hendley, Essex, UK) or four spot multiwell slides with 12-mm diameters (type PH005, Hendley) or Perkin-Elmer IS-PCR slides is recommended. Hendley slides are best used when performing IS-PCR/PCR-ISH with standard thermocyclers. With Hendley slides, the wells formed by the thin Teflon coating localizes the reagents so that minimal volumes can be used. It is recommended that slides are precoated with 3-amino-propyl-triethoxysilane to maximize section adherence. Perkin-Elmer slides come pretreated with silane.
2. 2% Decon 90: Dissolve by stirring 10 mL of Decon 90 in 490 mL of distilled water.
3. 2% 3-amino-propyl-triethoxysilane in acetone: In a fume hood, dissolve 10 mL of 3-amino-propyl-triethoxysilane in 100% acetone.

2.1.3. Preparation of Cultured Cells, Fresh Frozen Tissue, and Paraffin-Embedded Tissues

1 Phosphate-buffered saline (PBS) (Sigma, Poole, UK).
2. Methanol/acetic acid (3.1 [v/v]).
3. Fixatives:
   a NBF· Dissolve 4 g of sodium dihydrogen phosphate monohydrate and 6 5 g of disodium hydrogen phosphate anhydrous in 900 mL of distilled H_2O. To this, add 100 mL 40% formaldehyde.
   b 4% (v/v) Aqueous formalin
   c. 4% (v/v) Formaldehyde solution.
4 Cryostat
5. Ethanol
6. Xylene.

2.1.4. Tissue and Cell Pretreatment

1. 4% Paraformaldehyde in PBS· Dissolve 12 g of paraformaldehyde in 300 mL of PBS by heating to near boiling point then cool rapidly in ice prior to use.
2 0 1 *M* Tris-HCl, pH 7.2: Dissolve 24.2 g of Trizma base in 1600 mL of water and adjust the pH to 7 2 with HCl. Add distilled water to make up to 2 L
3 0 1 *M* Tris-HCl, pH 7.2, with 0.25% Triton X-100, 0.25% Nonidet P-40. To 1 L of 0.1 *M* Tris-HCl, pH 7 2, add 2.5 mL of Triton X-100 and 2 5 mL of Nonidet P-40. Warm the solution gently to dissolve the detergents and make up to 1 L.
4. 2X SSC: Make up a 20X SSC stock solution and dilute to 2X SSC when required To make up a 20X SSC stock solution, dissolve 175.2 g of sodium chloride and 88.2 g of sodium citrate in a liter of distilled water.
5 NBF (*see* **Subheading 2.1.3., step 3a**).
6. Proteinase K (Boehringer Mannheim, Mannheim, Germany): 0.1–0.5 mg/mL
7. Proteinase K buffer: 50 m*M* Tris-HCl, pH 7.6 and 5 m*M* ethylenediamine tetra-acetic acid (EDTA). Dissolve 6.05 g of Trizma base, 1.86 g of EDTA in 800 mL of distilled water and adjust to pH 7.6 with HCl. Adjust the final volume to 1 L with distilled water.
8 Glycerol
9 PBS containing 0.2% (w/v) glycine.
10. 0.1% (w/v) Sodium azide containing 0.3% hydrogen peroxide (v/v).
11 0.02 *M* HCl.
12. 0.01% Triton X-100 in PBS.
13. Terasaki plates

2.1.5. Amplification

The concentrations of some of the reagents in the amplifying solution for PCR ISH/IS-PCR vary compared with those for standard PCR. The optimum concentrations are as follows:

1. 25 m*M* Magnesium stock solution· final concentration 3 5–5.0 m*M*
2. 10X PCR buffer: 100 m*M* Tris-HCl pH 8.3, 500 m*M* KCl, 0 01% gelatin.
3. 10 m*M* Stock solutions of each of dATP, dCTP, dGTP, dTTP. final concentration 200 μ*M*.
4. Primers: final concentration 1–5 μ*M*.
5. 10 U/50 μL *Taq* DNA polymerase (*Taq* IS).
6. Amplification machine (*see* **Subheading 2.1.1.**)
7. Perkin-Elmer Assembly Tool, Perkin-Elmer AmpliCover Clip, Perkin-Elmer AmpliCover Disc (if the Perkin Elmer Gene Amp IS-PCR System 1000 is used)
8. Aluminium foil (if a standard thermal cycler is used).
9. 2% Paraformaldehye (for optional postfixing)
10. Gel bond (FMC Bioproducts, Rockland, ME)

The optimum concentrations of magnesium and *Taq* polymerase are greater than those for standard PCR. This reflects the difficulty of entry of these reagents at the site of DNA amplification and sequestration of magnesium by cellular components and/or nonspecific binding of *Taq* polymerase to the glass slide.

2.1.6. Probe Labeling

2.1.6.1. Nick Translation

1. Probe DNA.
2. 10X Nick translation buffer (10X NTB): 0.5 *M* Tris-HCl, pH 7 8, 50 m*M* $MgCl_2$, 0.5 mg/mL.
3. BSA (nuclease free)
4. 10X dNTPs: 0.5 m*M* dATP, dGTP, dCTP, dTTP with one triphosphate replaced by the labeled nucleotide at optimum concentration.
5. 100 m*M* dithiothreitol (DTT)
6. Digoxigenin-11-dUTP (DIG-dUTP) as the labeled triphosphate (Boehringer Mannheim).
7. 1 mg/mL DNase I stock.
8. 5 U/μL DNA polymerase I.
9. 20 mg/mL Glycogen.
10. 0.5 *M* EDTA, pH 8.0.
11. 3 *M* Sodium acetate, pH 5.6.
12. Sephadex G50 Column.
13. Carrier herring sperm DNA
14. TE: 10 m*M* Tris-HCl, pH 8 0, 1 m*M* EDTA.
15. Ethanol (–20°C)

2.1.6.2. Random Prime Labeling

1. Probe DNA.
2. 10X Hexanucleotide mixture (10X HM): 0.5 *M* Tris-HCl, pH 7.2, 0.1 *M* $MgCl_2$, 1 m*M* DDT, 2 mg/mL BSA, 62 5 A_{260} U/mL hexanucleotides.

3. 10X dNTPs: 0.5 m*M* dATP, dGTP, dCTP, dTTP with one triphosphate replaced by the labeled nucleotide at optimal concentration.
4. DIG-dUTP: as the labeled triphosphate (Boehringer Mannheim)
5. 2 U/mL Klenow enzyme
6. 0.5 *M* EDTA, pH 7.4.
7. 3 *M* Sodium acetate, pH 5.6
8. Sephadex G50 Column.
9. Carrier herring sperm; DNA.
10. TE 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
11. Ethanol (–20°C).

2.1.6.3. 3' and 5' Prime End Labeling of Oligonucleotide Probes

1. Probe DNA.
2. 10X Terminal transferase buffer (10X TTB): 1 *M* potassium cacodylate, 10 m*M* $CoCl_2$, 250 mM Tris-HCl, 2 mM DTT, pH 7.6, 2 mg/mL BSA.
3. 10 m*M* dATP.
4. DIG-dUTP: as the labeled triphosphate (Boehringer Mannheim).
5. Oligonucleotide.
6. Terminal transferase
7. 4 *M* LiCl
8. Ethanol (–20°C)
9. 0.2 *M* EDTA, pH 8.0.
10. TED buffer: 10 mM Tris-HCl, pH 8.0, 1 m*M* EDTA, 10 m*M* DTT.

2.1.6.4. 3' Prime End Tailing Method of Oligonucleotide Probes

1. Formamide
2. Sterile water
3. Dextran sulfate
4. 20X Sodium chloride, sodium citrate (SSC).
5. Oligonucleotide probe
6. 5X TTB: 0.5 *M* potassium cacodylate, 5 m*M* $CoCl_2$, 125 m*M* Tris-HCl, 1 m*M* DTT, pH 7 6, 1 mg/mL BSA.
7. 25 m*M* $CoCl_2$.
8. 10 m*M* dATP
9. 1 m*M* Digoxigenin dUTP.
10. Terminal transferase.
11. 3 *M* Sodium acetate
12. Ethanol.

2.1.7. Hybridization of DNA Probes to Retreated Tissue Sections

Four alternative hybridization buffers are recommended (*see* **Note 15**):

1. Hybridization buffer 1: 50 m*M* Tris-HCl, pH 7.2, 10% dextran sulfate, 2X SSC, 2X Denhardt's (i.e., 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA),

5 mg/mL single-stranded salmon sperm DNA, 50% formamide. Make up a stock solution of 2X hybridization buffer. Add 20 g of dextran sulfate to 25 mL of 200 m*M* Tris-HCl, pH 7.2, and heat with stirring to dissolve. Dissolve separately 0 04 g of Ficoll, polyvinylpyrrolidine, and BSA in 5 mL of 200 m*M* Tris-HCl, pH 7.2 by heating. Allow all the solutions to cool to room temperature and combine them with the cooled dextran sulfate solution. Add 3.5 g sodium chloride and 1.76 g sodium citrate to 10 mL of 200 m*M* Tris-HCl pH 7.2. Stir to dissolve and combine with the dextran sulfate/Denhardt's solution. Add 8 mL of a 5 mg/mL solution of single-stranded salmon sperm DNA in water Adjust the total volume of the buffer to 100 mL with distilled water Store this double-strength buffer at 4°C. For hybridization, take the stock buffer and add an equal volume of formamide to give buffer containing 50% formamide

2. Hybridization buffer 2 ("blotto buffer"): 2X SSC, 5% dextran sulfate, 0.2% (w/v) dried milk powder (pure, containing no vegetable extracts), 50% formamide. Dissolve 0.4 g of dried milk powder in 10 mL of distilled water. Add 10 g of dextran sulfate to 50 mL distilled water and stir to dissolve (heat gently if necessary) When cool add 20 mL of 20X SSC and the milk solution and make up to a final volume of 100 mL with distilled water. Add an equal volume of formamide to give a working strength of blotto buffer This hybridization buffer is easily made and gives results comparable to or better than buffer 1.
3. Hybridization buffer 3: This buffer is particularly useful when cocktails of genomic probes are used for the analysis of routine cervical smears for the detection of HPV 6, 11, 16, 18, 31, 33, and so on. The buffer contains 50% formamide, 5% dextran sulfate, 300 mL NaCl, 30 m*M* sodium citrate, 50 m*M* of Tris HCl, pH 7 4, 0 1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone (molecular weight 40,000), 0.2% (w/v) Ficoll, (molecular weight 400,000), 5 m*M* EDTA, 200 ng/mL sheared human DNA.
4. Hybridization buffer 4 (for oligo probes): Suitable buffers for oligo probes are as follows: 10% formamide (optional), 2X SSC, 5% dextran sulfate, 0.2% dried milk powder
5. Gel bond film (*see* **Note 16**).
6. Nail varnish (e.g., Boots No. 17, Boots, Nottingham, UK).
7. Baking tray.

2.1.8. Posthybridization Washing

1. 20X SSC: The final concentration can be adjusted in accordance to stringency required.
2. Water bath or oven to maintain washes at the appropriate temperature.

2.1.9. Detection of Hybridization Signal

2.1.9.1. Detection of Biotinylated Probes

One-step procedure:

1. Detection buffer 1: 0 1 *M* Tris-HCl, pH 7.5, 0.1 *M* NaCl, 2 m*M* $MgCl_2$, 0.05% Triton X-100.
2. Detection buffer 2: 0.1 *M* Tris-HCl, pH 9.5, 0.1*M* NaCl, 50 m*M* $MgCl_2$.

3. Tris-buffered saline (TBS): 50 m*M* Tris-HCl, 100 m*M* NaCl, pH 7 2
4. TBS/Triton X-100 (TBT) (blocking reagent): 50 m*M* Tris-HCl, 100 m*M* NaCl, pH 7.2, 3% (w/v) BSA, 0.5% Triton X-100 (v/v)
5. Development reagent (NBT/BCIP) for alkaline phosphatase detection: Make up fresh, just prior to use and keep in the dark, consisting of· 3.75 mL detection buffer 2, 16 5 mL nitro-blue tetrazolium (NBT) (75 mg/mL in dimethylsulfoxide), bromochloroindolyl phosphate (BCIP) (50 mg/mL in dimethylsulfoxide)
   The NBT solution is added to the detection buffer 2 and mixed gently by inversion before the BCIP is added.
6. Development reagent (3-amino-9-ethyl carbazole [AEC]) for peroxidase detection: This solution of peroxidase substrate uses a combination of H_2O_2 and AEC (Sigma). This is achieved by dissolving 2 mg of AEC in 1.2 mL of dimethylsulfoxide (Merk, UK) in a glass tube adding 10 mL of 20 m*M* acetate buffer, pH 5.0–5.2. After inverting the glass tube, add 1 mL 30% (v/v) hydrogen peroxide (Merk). Development occurs over 10–20 min.

Two-step procedure:

1 5% (w/v) BSA solution in water
2. Avidin DN (Vector, Burlingame, CA)
3 Biotinylated alkaline phosphatase (Vector)

Three-step procedure

1. Monoclonal mouse antibiotin (Dako, Santa Barbara, CA)
2. Biotinylated rabbit antimouse $F(ab')_2$ fragment (Dako).
3. Avidin alkaline phosphatase.
4. Streptavidin peroxidase (Dako)
5 Nonfat milk (e.g., Marvel, Darknau).

2.1.9.2. Detection of Probe with Gold-Labeled Goat Antibiotin

1. Lugol's iodine
2. 2.5% (w/v) Aqueous sodium thiosulfate
3. TBS containing 0.8% (w/v) BSA, 0.1% (w/v) gelatin, 5% (v/v) normal swine serum and 2 m*M* sodium azide.
4. 1 n*M* Gold-labeled goat antibody solution: 1:10 Auroprobe in TBS containing 0.1% (w/v) gelatin, 0.8% (w/v) BSA, 1% (v/v) normal swine serum, and 2 n*M* sodium azide.
5. Dark room with an S902 or F904 safelight.
6 In a dark room prepare a silver developing solution as follows:

500 g/L Gum acacia	7.5 mL diluted to 60 mL with water
Citrate buffer, pH 3.5	10 mL
0.85 g/15 mL Hydroquinone	15 mL
0.11 g/15 mL Silver lactate	15 mL

   The citrate buffer (pH 3.5) consists of 2–3.5 g of trisodium citrate dihydrate and 25.5 g citric acid monohydrate in 100 mL of distilled water.
7. Synthetic resin

2.1.9.3. Detection of Digoxigenin-Labeled Probes

1. Incubation tray.
2. Alkaline phosphatase-conjugated antidigoxigenin (Boehringer Mannheim).
3. TBT, NBT/BCIP, TBS (*see* above)

Three-step procedure:

1. Monoclonal antidigoxin (Sigma)
2. Biotinylated rabbit antimouse $F(ab')_2$ (Dako).
3. Avidin alkaline phosphatase or avidin/peroxidase.
4. Skim milk.
5. NBT/BCIP or AEC development reagent (*see* **Subheading 2.1.9.1.**)

Five-step procedure:

1. Monoclonal antibiotin (Dako).

2.1.9.4. Simultaneous Detection of Biotin and Digoxigenin-Labeled Probes

1. Materials as for the corresponding single label detection.

2.1.9.5. Fluorescent Probe Detection

1. Normal rabbit serum.
2. TBT, detection buffer 2, NBT/BCIP development buffer (*see* **Subheading 2.1.9.1.**)
3. Rabbit antifluorescein isothiocyanate (FITC) conjugated to alkaline phosphatase (Nova Castra, Newcastle, UK).

2.2. IS-PCR

1. Equipment: same as for PCR-ISH.
2. Amplification.
   a. Perkin-Elmer IS PCR core kit, including dNTP, 10X PCR buffer and Ampli*Taq* IS.
   b. Biotin-11-dUTP or DIG-11-dUTP or a primer labeled with a 5'-biotin or 5'-DIG
3. Detection: same as for PCR-ISH

2.3. RT IS-PCR

2.3.1. Preparation of Samples

1. Diethyl pyrocarbonate (DEPC)-treated water.
2. Precoated slides as for PCR-ISH.
3. Solvent for dewaxing: xylene and ethanol.
4. Trypsin or pepsin (2 mg/mL) or proteinase K(0.01–0 5 mg/mL) in proteinase K buffer (50 m*M* Tris-HCl, pH 7.6, 5 m*M* EDTA) (*see* **Note 2**)

2.3.2. DNase Digestion

1. 10X Digest buffer: 35 mL of 3 *M* sodium acetate, 5 mL of 1 *M* $MgSO_4$, and 60 mL distilled H_2O.

2 10 U/mL of RNase-free DNase (Boehringer Mannheim).
3. Autoclaved coverslips.

2.3.3. Using MMLV RT and Taq *DNA Polymerase: Two-Step Approach*

1. DNase.
2. RT-PCR kit (Perkin-Elmer).
3. Nail varnish.
4. Sterile mineral oil.
5. Xylene.
6. Ethanol.

2.3.4. Amplification

1. Perkin-Elmer GeneAmp Kit.
2. 2% BSA.
3 DIG-dUTP (10 m*M*)
4. PCR primers.
5. Coverslip.
6 Nail varnish
7 Mineral oil
8. Xylene.
9. Ethanol

*2.3.5. Reverse Transcription of RNA and PCR Amplification of cDNA Using EZ r*Tth *PCR Kit*

1. 5X EZ buffer: 250 m*M* bicine, 575 m*M* potassium acetate, 40% (w/v) glycerol, pH 8.2.
2. 25 m*M* Mn $(OAc)_2$ solution.
3. dNTPs (dATP, dCTP, dGTP and dTTP): one tube containing 3.2 μ*M* of each deoxynucleotide as a 10 m*M* solution.
4. Primers.
5. r*Tth* DNA polymerase: 2.5 U/μL in stored buffer.
6. Gene Amp PCR water (Perkin-Elmer) or autoclaved DEPC water that has been deionized.
7 Precoated slides from Perkin-Elmer.
8. 10% NBF (made up with formalin, pH 7.2).
9 PBS (Sigma)
10. 0.3 mg/mL Proteinase K in 0.1 *M* Tris-HCl, pH 7.2 with 0.25% Triton X-100
11. 0.1% Sodium azide/0.3% hydrogen peroxide.
12. RNase free DNase (Promega, Madison WI; 1 U/μL)
13. 10X Digestion buffer for RNase free DNase: 35 μL 3 *M* NaOAc, 5 μL *M* magnesium sulfate and 60 μL DEPC water.
14. Terasaki plates.
15 TBT
16. 2X SSC
17 TE.

18 Anti-DIG-conjugated peroxidase.
19. TBS.

3. Methods

3.1. PCR-ISH

3.1.1. Slide Preparation

Coating slides with APES:

1. Immerse slides in 2% (v/v) Decon 90 in warm water for 30 min
2 Wash thoroughly in distilled water to remove the detergent
3 In a fume hood, drain off excess water and immerse in 100% acetone for 1–2 min
4. In a fume hood, drain off acetone and immerse in 2% APES in acetone for 5 min
5 Drain off excess solution and wash in running water for 1–2 min
6 Drain off excess water and allow slides to dry overnight at room temperature

3.1.2. Preparation of Culture Cells, Fresh Frozen Tissue, and Paraffin-Embedded Tissues

3.1.2.1. Preparation of Culture Cells

1 Wash cultured cells three times with sterile PBS at 4°C to remove all traces of culture medium.
2. Resuspend at a concentration of 2×10^6 cells/mL and pipet 50 µL on to each well of precoated slides.
3 Allow the cells to adhere for 10 min, remove any excess, then fix immediately

3.1.2.2. Preparation of Cervical Smears

1. Incubate routine cervical smear slides in methanol/acetic acid (3·1 [v/v]) for 10 min at 22°C.
2. Fix immediately as for cultured cells (*see* **Note 4**).

3.1.2.3. Preparation of Cryostat Sections of Fresh Frozen Tissue

1. Cut 5-µm sections onto each well of precoated slides.
2 Wash twice in sterile PBS at 4°C to remove all traces of the embedding medium (OCT compound). This material binds many of the detection reagents that normally result in very high background
3 Fix sections immediately as for cultured cells.

3.1.2.4. Preparation of Fixed Paraffin-Embedded Tissues (*see* **Note 5**)

1. Fix tissue in 10% (v/v) aqueous formalin or 4% (v/v) formaldehyde solution or in (NBF) prior to paraffin wax embedding.
2. Cut sections onto slides
3. Incubate sections on a hot plate overnight in order to achieve maximum section adhesion (*see* **Note 6**)

4. Carry out section dewaxing by entirely immersing the slides in xylene at 37°C for approx 30 min followed by xylene at room temperature for a further 10 min.
5. Immerse the slides in 100% ethanol at room temperature for another 10 min.
6. Transfer slides into fresh absolute ethanol before rehydrating through a graded ethanol series to water over a 10-min period. The pretreatment steps are then performed as described in **Subheading 3.1.3.3.**

3.1.3. Tissue and Cell Pretreatment

3.1.3.1. Pretreatment of Fresh Cell, Cell Smears, and Cryostat Sections

1 Fix the cells in 4% paraformaldehyde in PBS or NBF for 30 min.
2 Rinse the slides in PBS for 5 min.
3. Immerse the slides in 0 1 *M* Tris-HCl pH 7.2 containing 0.25% Triton X-100 and 0.25% Nonidet P40, twice for 5 min (*see* **Note 7**). Additional mild proteolysis may also be required (i.e., 0.1 mg to 0 5 mg/mL proteinase K)
4 Immerse the slides in 0 1 *M* Tris-HCl, pH 7.2, twice for 5 min.
5 Immerse the slides in 20% (v/v) aqueous acetic acid at 4°C for 15 s (*see* **Note 8**).
6. Wash the slides in 0.1 *M* Tris-HCl, pH 7.2, three more times for 5 min.
7 Incubate the slides in aqueous 20% glycerol for 30 min at room temperature
8 Rinse with 0 1 *M* Tris-HCl, pH 7.2.
9 Immerse the slides in 2X SSC for 10 min.

3.1.3.2 Pretreatment of Cervical Smears

A specialized method is presented here for pretreatment of cervical smears for use with a peroxidase detection system.

1. Fix in 4% paraformaldehyde in PBS for 15 min at 22°C.
2 Wash in PBS containing 0.2% (w/v) glycine for 5 min.
3. Rinse the slides in PBS.
4. Immerse slides in 0.1% (w/v) sodium azide containing 0.3% hydrogen peroxide (v/v) for 10 min (*see* **Note 9**).
5. Wash slides in PBS for 5 min.
6 Incubate the slides in proteinase K solution (1 mg/mL) in PBS for 15 min at 37°C
7 Wash in PBS for 5 min
8. Immerse slides in 4% paraformaldehyde or NBF for 5 min.
9. Wash in PBS glycine for 5 min.
10 Wash in PBS for 5 min.
11. Air-dry at 37°C.

3.1.3.3. Pretreatment of Formalin-Fixed Paraffin-Embedded Tissues

Method 1:

1 Immerse the prepared slides in 0.02 *M* HCl for 10 min (*see* **Note 10**).
2. Wash twice in PBS for 5 min each.

3 Extract with 0.01% Triton X-100 in PBS for 3 min *(optional)*
4. Wash twice in PBS for 5 min each.
5. Equilibrate the slides in prewarmed proteinase K buffer at 37°C for 10 min (*see* **Note 11**).
6. Incubate the slides with proteinase K at 37°C for 10–20 min in a Terasaki plate. The optimum concentration of proteinase K (0 1–5 mg/mL) and the time of digestion will be dependent on the tissue.
7 Wash the slides in two changes of PBS containing 2 mg/mL of glycine for 5 min each This inhibits the action of the proteinase K *(optional)*.
8. Immerse the slides in aqueous 20% acetic acid at 4°C for 15 s to block endogenous alkaline phosphatase activity. When using a peroxidase detection system, peroxidase block is carried out using 0 1% (w/v) sodium azide containing 0 3% hydrogen peroxide.
9 Wash in two changes of PBS for 10 min each
10. Dehydrate through a fresh-graded ethanol series

Method 2: (This is a quicker protocol than Method 1, but cellular architecture preservation is not as reliable).

1. Spot proteinase K in PBS onto the prepared tissue section and place in either a preheated oven at 37°C for 10 min or place into a Terasaki plate floating in a water bath at 37°C for 15 min. The optimum concentration of proteinase K (0 1–0.5 mg/mL) and the time of digestion will be dependent on the tissue
2 Wash in distilled water then air dry at 75°C

Method 3: (This method originates from Lewis et al., personal communication, and is currently being evaluated).

1 Proceed with **Method 1, steps 1–3**.
2. Immerse the slides in 0.01% Triton X-100 in PBS for 90 s.
3. Rinse slides in PBS for 2 min.
4. Transfer the slides into a Coplin jar containing 10 µg/mL proteinase K in 0.1 *M* Tris-HCl, pH 7.5, 5 m*M* EDTA, and place the jar in a microwave oven Use five slides in 50 ml of buffer per digestion.
5. Microwave the slides in the Coplin jar on full power (800 w) for 12 s. (This should raise the temperature of the solution to 37°C) Leave the Coplin jar in the oven
6 Pulse heat the Coplin jar at 10-min intervals for 5 s at full power. The temperature gradually increases to a level not greater than 50°C.
7. After 30 min, microwave the Coplin jar for 1 min, in order to boil the contents. Remove the jar from the oven Care should be taken, because the jar is extremely hot
8. Do not cool the Coplin jar, because this will cause shattering of the glass. Pour off the hot buffer and transfer the slides to another Coplin jar. Pour on 20% acetic acid at 4°C and incubate the slides for 15 s
9. Transfer the slides to PBS and rinse for 2 min.
10. Transfer the slides to fresh molecular biology grade water for 2 min and then dehydrate through graded ethanols to fresh 100% ethanol.
11. The slides can be stored in 100% ethanol until required.

3.1.4. Amplification

Method 1 is based on the Perkin-Elmer Gene Amp IS PCR Core Kit protocol using the Gene Amp IS PCR system 1000.

1. Set up the reaction mixture using the the proportions listed in **Table 1**, with the exception of the Ampli*Taq* DNA polymerase IS, giving a total volume of 50 µL. The solution is prepared in 1.5 mL Eppendorf tubes.
2. Turn on the Perkin-Elmer Gene Amp IS PCR System 1000 and start a SOAK file at 70°C. Plug in the Assembly Tool. Turn on a heat block and allow it to reach 70°C
3. Put the reaction mix tubes into the 70°C heat block and incubate for 5 min. After the 5-min incubation, add the appropriate amount of Ampli*Taq* DNA polymerase IS to each reaction mix tube, mix gently, and return the tube to the heat block If the primer composition of each tube is different, be careful not to cross-contaminate the mixtures.
4. Lay an AmpliCover Clip™ on a clean surface so that the oval recess is face up. Gently slide the grips to the open position. Place an AmpliCover Disc, with the tabs down, into the recess of the AmpliCover Clip Try to avoid pushing the center of the AmpliCover Disc Repeat this step until enough AmpliCover Clip/AmpliCover Disc assemblies are prepared to accommodate all of the samples in the experiment.
5. Place a prepared slide on the Assembly Tool platform surface Position the slide so that the first sample is over the inscribed oval on the platform. The samples must be loaded in consecutive order, starting with the sample furthest away from the frosted end of the slide
6. Place an AmpliCover Clip and AmpliCover Disc assembly into the magnetic slot in the Assembly Tool arm. Make sure the assembly is correctly aligned in the slot and the grips are in the open position.
7. Dispense 50 µL of the heated reaction mix directly onto the sample spot, being careful not to touch the sample with the pipet tip. Pipet gently, forming a bead of reaction mix over the sample Pull the pipet tip away from the slide while delivering the solution to form as tall a bead as possible. Avoid forming bubbles on the bead surface by not expelling air from the pipet
8. Lower the arm of the Assembly Tool and press gently until it latches Slowly turn the handle with a smooth motion until it stops. Do not reverse direction after the AmpliCover Disc has made contact with the reaction mix Press the two levers on top of the Assembly Tool arm to engage the grips onto the edges of the slide. Release the levers, disengage the handle, and raise the arm of the tool.
9. Move the slide along the platform surface until the second sample is in the loading position and repeat **steps 5–7** Finally, load the third sample.
10. Grip the slide by the edges of the frosted label area and lift it from the platform surface.
11. Open the sample block cover of the preheated (70°C) instrument and raise slide retaining lever. Place the slide into one of the vertical slots of the sample block This slide can only fit in one orientation (*see* User Manual for the Perkin-Elmer Gene Amp IS PCR System 1000 for further details). Lower the retaining lever and close the block cover.

Table 1
Components Required for PCR-ISH

Components	Volume (μL/50 μL total Rxn vol.)	Final concentration
Water (add last)		
10X PCR buffer II	5	1X
10 m*M* dATP	1	200 μ*M*
10 m*M* dCTP	1	200 μ*M*
10 m*M* dGTP	1	200 μ*M*
10 m*M* dTTP	1	200 μ*M*
Primer 1	0.5–2 5	0.2–1.0 μ*M*
Primer 2	0.5–2 5	0.2–1 0 μ*M*
25 m*M* $MgCl_2$	2– 9	1–4 5 m*M*
Ampli*Taq* DNA polymerase IS	0.5	10 U
Total	50 μL	

12. Repeat the loading sequence with the remaining slides
13. Commence thermal cycling using a typical cycling program of 94°C for 1 min followed by 94°C for 55 s; 55°C for 45 sec. Then cool the slides at 4°C (*see* **Note 12**)

Method 2 (using a standard thermal cycler): This procedure can be simplified by following the dewaxing steps in the protocols from the previous section and then by proceeding directly to the proteinase K digestion

1. The slide containing the fixed cell suspension is placed in an aluminium foil boat, trimmed to slightly larger proportions than the slide
2. 10 μL of PCR solution consisting of 1X PCR buffer, 4 5 m*M* $MgCl_2$, 0.01% gelatin, 200 μ*M* of each dNTP and 5 μ*M* primer is placed on top of the cell suspension contained in the deep well slide. The well is then covered with a precut piece of Gel bond (FMC Bioproducts), hydrophobic side down.
3. The slide (in the aluminium foil boat) is placed on the heating block of the thermal cycler, and the temperature allowed to increase to 80°C.
4. Once the temperature has reached 80°C, a corner of the gel bond is lifted and 2.5 μL *Taq* DNA polymerase (2.5 U/12.5 μL) are added to the PCR mix contained on the slide
5. Replace the coverslip and seal the margins with nail polish. Place 1–2 mL of pre-heated mineral oil (80°C) on top of the slide to ensure optimum thermal kinetics Use a thermal profile of: 94°C for 6 min, 40 cycles of 55°C for 2 min, and 94°C for 1 min.
6. Following amplification, the slide is dipped in chloroform to remove mineral oil and the gel bond coverslip carefully removed. Gel bond hydrophobic side toward the cell suspension allows the PCR reagent mix to remain on the tissue in the well.
7. Carefully dip the slides in 100% ethanol and dehydrate. The slides can also be postfixed in 2% paraformaldehyde to maintain localization of PCR product

8. Proceed with the hybridization of biotin- or digoxigenin-labeled probes to amplified DNA (detailed in **Subheading 3.1.5.**).
9. Detect the hybrid using the appropriate protocol. Shorter detection protocols are preferred.

3.1.5. Probe Labeling

3.1.5.1. Nick Translation (from Hopman et al. *[18]*)

1. To one Eppendorf tube add: 1 µg DNA, 5 µL 10X NTB, 5 µL 10X dNTPs, 5 µL 0.5 µM labeled triphosphate, 5 µL 100 mM DTT, 5 µl 1 mg/µL DNase I, 5 µL DNA polymerase I, and make up the final volume to 50 µL with distilled H_2O.
2. Mix and incubate at 15°C for 2 h.
3. Purify the labeled probe by Sephadex G-50 chromatography (column previously equilibrated with TE).
4. Add 1 µL glycogen or carrier DNA to the eluate and precipitate with ethanol by adding 1/10 vol of 3 *M* sodium acetate, and 2.5 vol of the ethanol.
5. Incubate at –20°C for 1 h and centrifuge at 15,000*g* for 30 min at 4°C.
6. Dry the pellet and redissolve with TE.

3.1.5.2. Random Prime Labeling (from Hopman et al. *[18]*)

This is performed on a linearized, single-stranded DNA template. The oligonucleotides are annealed randomly and then conjugated with labeled nucleotides. In comparison with nick translation, the probes labeled by random priming are composed of a larger portion of small fragments giving a greater incorporation of the label.

1. Linearize and denature 10 ng to 3 µg of DNA by heating for 10 min at 95°C; then chill on ice for 5 min.
2. To one Eppendorf tube on ice, add 2 µL of 10X HM (1 m*M*), 2 µL of 10X dNTPs, 2 µL of labeled triphosphates (0.5 m*M*), 10 ng to 3 µg of DNA, and then make up the final volume to 19 µL with dH_2O.
3. Add 1 µL (2 U) Klenow enzyme.
4. Mix, microcentrifuge briefly, and incubate for at least 60 min at 37°C.
5. Stop the polymerase reaction by adding 2 µL 400mM EDTA, pH 7.4.
6. Precipitate the labeled DNA by adding 1/10 vol of 3 *M* sodium acetate, and 2 5 vol of ethanol.
7. Keep at –20°C for 2 h and centrifuge at 15,000*g* for 30 min at 4°C
8. Wash the pellet with cold ethanol, carefully remove the solvent, and dissolve the pellet in TE buffer. Store at –20°C until use.

3.1.5.3. 3'- and 5'-prime end labeling of oligonucleotide probes (from Hopman et al. *[18]*)

1. To one Eppendorf tube, add 100 p*M* of oligonucleotide, 2 µL of 10X TTB, 1 µL of dATP, 1 µL of labeled triphosphate, 1 µL of terminal transferase (25–50 U), and then make up the final volume to 20 µL with dH_2O.

2. Incubate for 1 h at 37°C.
3. Stop the polymerase reaction by addition 2 µL 0.2 *M* EDTA, pH 8 0.
4. Precipitate the labeled DNA by adding 2 5 µL 4 *M* LiCl and 75 µL –20°C ethanol
5 Keep at –20°C for 2 h and centrifuge at 15,000*g* for 30 min at 4°C
6. Wash the pellet with cold ethanol, carefully remove the supernatant, and dissolve the pellet in TED buffer. Store at –20°C until use (*see* **Note 13**).

3.1.5.4. 3' PRIME END TAILING METHOD OF OLIGONUCLEOTIDE PROBES (FROM NUOVO *[3]*)

The advantages of using DIG-tailed oligonucleotides as hybridization probes is the increased sensitivity owing to the incorporation of the several, small single-stranded DIG-nucleotides. The following method is performed with the DIG 3' primed oligonucleotide tailing kit from Boehringer Mannheim.

1. Assemble the probe cocktail for the oligonucleotide: 10 µL formamide, 39 µL of sterile water, 40 µL 25% dextran sulfate, 10 µL 20X SSC, 1 µL of the oligonucleotide probe.
2. Set up the reaction mixture: 4 µL 5X terminal transferase reaction buffer, 4 µL of 25 m*M* $CoCl_2$, 1 µL of 10 m*M* dATP, 1 µL of 1 µ*M* digoxigenin dUTP, 9 µL of 100 pmol/50 µL oligonucleotide, 1 µL terminal transferase.
3 After synthesis, precipitate the labeled DNA by adding 1/10 vol of 3 *M* sodium acetate, and 2.5 vol of the ethanol.
4. Keep at –20°C for 2 h and centrifuge at 15,000*g* for 30 min at 4°C.
5. Wash the pellet with cold ethanol, carefully remove the solvent, and dissolve the pellet in 20 µL distilled H_2O buffer. Store at –20°C until use

3.1.6. Hybridization of Labeled Probes to Pretreated Tissue Sections

1. Reconstitute probe (*see* **Note 14**) in the appropriate hybridization buffer (*see* **Note 15**). For Perkin-Elmer slides, apply 30 µL of genomic probe (2 ng/µL) to each section For PH106 Hendley slides, apply 75 µL of the appropriate genomic probe (200 ng/ml) to each section. For multiwell slides (PH005, Hendley), apply 8–10 µL of genomic probe (2 ng/µL). For oligoprobes apply 20–30 µL of probe (5–10 pmol/100 µL). For cervical smears, use hybridization buffer 3 (above) and a cocktail of probes (HPV 6, 11, 16, 18, 31, 33) at a concentration of 2 ng/µL of each probe.
2. Cut gel bond films to coverslip size and place hydrophobic side down over each section (*see* **Note 16**). Seal the gel bond in place with nail varnish For multiwell slides, a plastic coverslip may be used and it is advisable not to seal with nail varnish.
3. Place the slide onto a preheated baking tray and incubate at 90–95°C for 10 min (*see* **Note 17**).
4. Transfer slides to a humidified box and incubate slides at 37–42°C overnight or at 42°C for 2 h (*see* **Note 18**).

3.1.7. Posthybridization Washing

3.1.7.1. Washing with Increasing Stringency (*see* **Note 19**)

3.1.7 1.1.Genomic Probes. Two sequential washing procedures are described giving medium to high stringency that is sufficient for detection of specific hybridizations using genomic DNA probes. They can be adapted to the required stringency by varying the SSC concentrations and the temperature.

Method 1·

1. Remove the gel bond coverslips with a scalpel blade.
2. Wash the slides in SSC with agitation according to the following protocol·
   a. 2X SSC at room temperature for 10 min.
   b. 2X SSC at 60°C for 20 min.
   c 0.2X SSC at room temperature for 10 min.
   d 0.2X SSC at 42°C for 20 min
   e. 0.1X SSC at room temperature for 10 min.
   f. 2X SSC at room temperature for 1–2 min.

Method 2:

1 Wash slides according to the following protocol·
   a Fresh 4X SSC at 22°C for 5 min.
   b. Fresh 4X SSC at 22°C for 5 min.
   c Fresh 4X SSC at 22°C for 5 min.

3.1.7.1 2. Oligonucleotide probes With oligonucleotide probes (oligo-probes), titration experiments are required to optimize the conditions for which the signal is visible and when it disappears. Essentially this establishes the T_m for the particular probe.

1. Remove the gel bond coverslips with a scalpel blade.
2. Wash the slides in SSC with agitation according to the following protocol·
   a. 2X SSC at room temperature for 5 min.
   b. 0 2X SSC at 42°C for 20 min.
   c. 0.1X SSC at 45°C for 10 min.
   d. 0.1X SSC at 55°C for 10 min.

3.1.8. Detection of Hybridization Signal

Many detection techniques are now available for the detection of biotinylated- and DIG-labeled human and viral DNA probes. For convenience purposes, these are described as one-step, two-step, three-step, and five-step procedures, depending on the number of reagents used and steps involved in the detection protocol. In addition, the use of immunogold silver staining (IGSS) for the detection of biotinylated probes is also described.

3.1.8.1. Detection of Biotinylated Probes

The following four protocols describe different detection protocols for biotinylated probes. Remember the choice of detection systems depends on the level of sensitivity required.

One-step procedure:

1 Immerse the slides following the posthybridization washes in TBT (blocking reagent) at 22°C for 10 min
2. Transfer the slides to an incubation tray. Transfer a single slide at a time in order to prevent dehydration.
3 Incubate slides in avidin/alkaline phosphatase or avidin peroxidase (Dako), diluted 1/100 in TBT
4 Remove unbound conjugate by washing for 5 min twice in TBS
5 Incubate slides in NBT/BCIP or AEC development reagent for 10–30 min and monitor color development.
6 Terminate the color development reaction by washing in distilled water for 5 min

Two-step procedure:

1. Transfer the slides from the posthybridization washes into detection buffer 1 containing 5% (w/v) BSA and incubate at room temperature for a minimum of 30 min
2. Wipe excess buffer from the slides and transfer them to a slide incubation tray Transfer small numbers to try and prevent sections from drying out.
3 Add a few drops of detection buffer 1 containing Avidin DN (Vector) at a concentration of 10 µg/mL and incubate at room temperature for 10 min.
4. Wash the slides with agitation with two changes of buffer 1 for 10 min each
5 Return the slides to the incubation tray and add a few drops of buffer 1 containing biotinylated alkaline phosphatase (Vector) at a concentration of 10 µg/mL to each section. Incubate at room temperature for 10 min.
6 Wash the slides with agitation twice with detection buffer 1 for 10 min each.
7 Transfer the slides into detection buffer 2 and allow to equilibrate for 30 min
8. Return the slides to the incubation tray and cover the sections with NBT/BCIP development reagent Monitor the development of the color after 5 min and then continuously until development looks complete.
9 Terminate the reaction by immersion of slides in PBS for 5 min or distilled water

Three-step procedure:

1. Immerse the slides from the posthybridization washes in TBT at 22°C for 10 min
2. Transfer the slides to an incubation tray and incubate in monoclonal mouse antibiotin (Dako) diluted 1/50 in TBT.
3. Wash the slides twice in TBS for 10 min each.
4. Incubate slides in biotinylated rabbit antimouse $F(ab')_2$ fragment (Dako) diluted to 1 in 20 in TBT
5 Wash twice in TBS for 5 min.

6 Incubate slides in either avidin alkaline phosphatase diluted to 1 in 50 in TBT, or streptavidin peroxidase (Dako) diluted to 1 in 100 in TBT containing 5% nonfat milk.
7. Wash in TBS for 5 min
8. Incubate slides in either NBT/BCIP development reagent or AEC for 10–30 min, as appropriate.
9. Terminate the color development reaction by washing in distilled water for 5 min

3.1 8.2. Detection of Probe with Gold-Labeled Goat Anti-Biotin

1 Transfer the slides from the posthybridization washes into Lugol's iodine for 2 min. Rinse with TBS, decolorize in 2.5% (w/v) aqueous sodium thiosulfate, and wash in TBS twice for 5 min
2. Immerse the slides in TBS containing 0.8% (w/v) BSA, 0.1% (w/v) gelatin, 5% (v/v) normal swine serum, and 2 m*M* sodium azide for 20 min
3 Remove excess buffer and transfer slides from the incubation tray. Apply 1 n*M* gold-labeled goat antibody solution and incubate for 2 h
4 Wash sections in TBS with agitation twice for 5 min.
5 In a darkroom prepare the silver developing solution
6. Immerse the slides in the developing solution in the dark (use an S902 or F904 safelight), until the sections appear optimally developed, when viewed by light microscopy (3–10 min)
7 Wash in running tap water for 5 min.
8 Fix in 2.5% (w/v) aqueous sodium thiosulfate for 3 min.
9 Wash in running tap water for 1 min, counterstain as required, dehydrate, clear, and mount with synthetic resin.

3 1.8.3. Detection of DIG-Labeled Probes

The following three protocols describe detection techniques for DIG-labeled probes. The choice of detection system depends on the level of sensitivity required—the greater the number of steps, the more sensitive the technique.

One-step procedure:

1. Immerse the slides following the posthybridization washes in TBT (blocking reagent) at 22°C for 10 min
2. Transfer slides into an incubation tray.
3. Incubate sections in alkaline phosphatase-conjugated anti-DIG (Boehringer) diluted in 1/600 in TBT
4. Wash in TBS for 5 min, twice.
5. Develop the signal using NBT/BCIP development reagent for 10–30 min and monitor color development.
6. Terminate the color development reaction by washing in distilled water for 5 min.

Three-step procedure:

1. Immerse the slides from the posthybridization washes in TBT (blocking reagent) at 22°C for 10 min.

2 Transfer slides to an incubation tray
3 Incubate slides in monoclonal anti–DIG (Sigma) diluted in 1/10000 in TBT.
4. Wash in TBS twice for 5 min.
5. Incubate in biotinylated rabbit antimouse $F(ab')_2$ (Dako) fragment diluted to 1 in 200 in TBT.
6. Wash in TBS twice for 5 min.
7. Incubate the slides in avidin alkaline phosphatase, diluted to 1 in 50 in TBT or avidin/peroxidase diluted to 1 in 75 in TBT containing 5% (w/v) skim milk
8 Wash in TBS for 5 min.
9. Incubate the slides in NBT/BCIP or AEC development reagent as appropriate
10. Terminate the color development reaction by washing in distilled water for 5 min

Five-step procedure:

1. Follow the first six steps of the three-step protocol, incubate slides in monoclonal antibiotin (Dako) diluted to 1 in 50 in TBT.
2 Wash slides in TBS for 5 min.
3 Incubate slides in biotinylated rabbit antimouse $F(ab')_2$ fragment (Dako) for 10 min
4 Wash in TBS for 5 min.
5 Incubate the slides in avidin peroxidase diluted to 1 in 75 in TBT containing 5% (w/v) skim milk.
6. Incubate the slides in the AEC development reagent for 10–30 min and monitor color development.
7. Terminate the color development reaction by washing in distilled water for 5 min.

3.1.8.4 Simultaneous Detection of Biotin- and DIG-Labeled Probes

Simultaneous detection of two different nucleic acids can be achieved in the one tissue section using combined application of biotin- and DIG-labeled probes to their respective targets in the sample.

1. Incubate the slides at 22°C for 30 min in a mixture of streptavidin/peroxidase conjugate, diluted to 1 in 100 in TBT and alkaline phosphatase-conjugated anti-DIG, diluted to 1 in 600 in TBT.
2. Remove unbound conjugate by washing twice in TBS for 5 min.
3. Incubate in AEC development reagent for 30 min at 22°C
4. Terminate the reaction by thoroughly washing in TBS.
5. Wash in buffer 2 for 10 min.
6. Incubate in NBT/BCIP development reagent for 20–40 min.
7. Terminate the reaction by washing in distilled water for 5 min
8. Air dry the slides at 42°C and mount in glycerol jelly.
9. Following detection of the hybridization product, counterstain the section with an aqueous stain In the case of alkaline phosphatase (NBT/BCIP) detection, use 2% (w/v) methyl green and mount with glycerol jelly For AEC-detected slides, counterstain progressively with hematoxylin for approx 10–15 s and then mount in glycerol jelly.

3.1.8.5. Fluorescent Probe Detection

1. Place slides in an incubating tray and cover the sections with 100 mL of normal rabbit serum diluted to 1 in 50 in TBT and incubate 10 min.
2. Remove the slide, carefully, and add rabbit anti-FITC conjugated to alkaline phosphatase (Nova Castra) diluted to 1:100 or 1:200 in TBT. Incubate for 30 min.
3. Wash slides in TBS twice for 3 min
4. Wash the slides in alkaline phosphatase substrate buffer (detection buffer 2) for 5–10 min.
5. Incubate the slides in NBT/BCIP developer reagent for 10–15 min as appropriate.
6. Terminate the color development by washing in distilled H_2O for 5 min.

3.2. IS-PCR

3.2.1. Preparation of Samples

The preparation of samples is the same for PCR ISH.

3.2.2. Pretreatments

As with PCR-ISH, IS-PCR requires a number of precise manipulations if the method is to be successful. Unlike PCR-ISH, this method is not reliant on stringent washing postamplification for specificity. Too much pretreatment will result in the product leaking into the reaction components, whereas too little will not allow the reaction components into the cells.

3.2.3. Amplification

3.2.3.1. Magnesium Concentration Optimization Experiment

1. Dry down 7 × 50–100 pmol each of the primers in a 1.5-mL Eppendorf tube.
2. When dry, add the components of the IS kit as follows (make sufficient amounts for seven sections): 7 µL dATP, 7 µL dCTP, 7 µL dGTP, 7 µL dTTP, 5 µL 10X PCR, buffer 3, 2.1 µL Biotin-11-dUTP, or 7 µL DIG-11-dUTP (1 m*M*), and 3.5 µL AmpliTaq IS.
3. Mix thoroughly to dissolve the primers and transfer 9.8 µL of the master mix into six tubes.
4. Label the tubes 3, 4, 5, 6, 7, and 8 to correspond to the volume of magnesium chloride to be added to each tube and the corresponding volumes of magnesium chloride from the stock tube in the kit.
5. Make up the volume to 50 µL in each tube with distilled water by adding 37.2, 36.2, 35.2, 34.2, 33.2, and 31.2 µL, respectively. Cap the tubes and store on ice or at 4°C until required

3.2.3.2. Typical IS-PCR Set-Up (Using a Labeled Oligonucleotide or Labeled Primer)

1. The reaction mixture is prepared by adding the reagents in the proportions shown in **Table 2**, with the exception of the Ampli*Taq* DNA polymerase IS, giving a total volume of 50 µL The solution is prepared in 1.5-mL Eppendorf tubes.

2 Place the slides in the preheated assembly tool and apply the first 50 µL reaction mixture to the first section on the slide (place the slide into the assembly tool with the writing area on the right). Enclose the reaction mix onto the section with the clip and cover and slide the glass slide forward into the next position Repeat the process until all the sections have been assembled and then transfer the slide into the PCR machine held at 70°C.
3. Cycle the slides as for PCR ISH
4. At the end of the cycling disassemble the slides and transfer into 2X SSC briefly
5. Wash slides in PBS for 2 min then transfer into 5% BSA in detection buffer 1 for 30 min.

3.2.3. Detection of Signal

The detection of the results is the same for PCR ISH.

3.3. RT IS-PCR

All solutions for RT IS-PCR or RT PCR-ISH are made up in DNase and RNase treated solutions where possible. Standard washing buffers are made up in DEPC-treated water.

3.3.1. Preparation of Samples

1. 3 × 5-µm Tissue sections are placed onto precoated slides
2 The sections are dewaxed using xylene and 100% ethanol and then air dried
3 Digest the section with trypsin or pepsin (2 mg/mL) at RT for up to 90 min or alternatively with proteinase K (0.01–0.5 mg/mL) in proteinase K buffer.
4. Inactivate the protease with a 1 min wash in distilled H_2O followed by 100% ethanol for 1 min.

3.3.2. DNase Digestion

1. Digest two of three of the sections with RNase-free DNase. To each section add 10–40 µL of solution containing 1 µL of 10X digestion buffer, 1 µL of 10 U/mL RNase-free DNase, 8 µL of H_2O.
2 Cover the solution on the section with autoclaved coverslips and place in a humidity chamber at 37°C initially for 2 h
3 Then repeat the procedure again overnight.
4. Remove the coverslip, wash for 1 min in dH_2O and 100% ethanol, and air-dry

3.3.3. Using MMLV RT and Taq DNA Polymerase: Two-Step Approach

3.3.3.1. Reverse Transcription

1. Onto one of the two sections treated with DNase, add 10 µL of a mixture consisting of the following reagents found in the RT-PCR kit. 2 µL $MgCl_2$ (25 m*M*), 1 µL RT buffer, 1 µL dATP (10 m*M*), 1 µL dCTP (10 m*M*), 1 µL dGTP (10 m*M*), 1 µL dTTP (10 m*M*), 1 5 µL dH_2O, 0.5 µL 3'primer (20 m*M*), 0.5 µL RNase inhibitor, and 0.5 µL RT.

Table 2
Reagent Composition for IS-PCR

Components	Volume (μL/50 μL total Rxn vol.)	Final concentration
Water (add last)		
10X PCR buffer II	5	1X
10 m*M* dATP	1	200 μ*M*
10 m*M* dCTP	1	200 μ*M*
10 m*M* dGTP	1	200 μ*M*
10 m*M* dTTP	1	200 μ*M*
Biotin 11-dUTP or	0.3	
DIG-11-dUTP or	1	20 μ*M*
Primer 1 (labeled with biotin/DIG)	0.5–2.5	0.2–1.0 μ*M*
Primer 2	0.5–2.5	0 2–1.0 μ*M*
25 m*M* $MgCl_2$	2– 9	1–4.5 m*M*
Ampli*Taq* DNA polymerase IS	0.5	10 U
Total	50 μL	

2. To prevent drying the coverslips, anchor with one small drop of nail varnish, load onto a thermal cycler, incubate at 42°C for 30 min and cover with sterile mineral oil.
3. Remove coverslip and oil with a 5 min wash in xylene, followed by a 5 min wash in 100% ethanol.

3.3.3.2. Amplification

1. For each slide prepare a 25-μL solution containing the following· 2.5 μL PCR Buffer (from the Gene Amp Kit), 4.5 μL $MgCl_2$ (25 m*M*), 4.0 μL dNTP solution (final concentrate 200 μ*M* from kit protocol), 1.0 μL 2% BSA, 0 4 μL DIG-dUTP final conc 10 μ*M*, 1 μL primer 1 (20 m*M*), 1 μL primer 2 (20 μ*M*), 11 μL dH_2O, 0.6 μL *Taq* polymerase.
2. Add to slide and cover with one large coverslip anchored with two drops of nail varnish.
3. Ramp to 80°C, add preheated mineral oil, go to 94°C for 3 min.
4. Cycle at 55°C for 2 min, 94°C or 1 min × 15–20 cycles.
5. Remove the coverslip and polish with scalpel, then wash for 5 min in xylene and 5 min in 100% ethanol and air-dry.

3.3.4. Reverse Transcription of RNA and PCR Amplification of cDNA Using EZ rTth PCR Kit

1 Precoated slides from Perkin-Elmer should be used, sections are fixed in 10% NBF (made up with formalin, pH 7.2)
2. Rinse slides twice in PBS for 5 min.

3. Allow sections to air dry on the bench.
4. Incubate the slides with proteinase K at 37°C.
5. Wash the slides in 0 1 *M* Tris-HCl (pH 7.2) three times
6. Block endogenous peroxidase in a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 10 min.
7. Wash the slides twice in 0.1 *M* Tris-HCl (pH 7 2).
8. Perform RNase free DNase digestion (Promega; 1 U/μL) in a moist Terasaki plate (10 U/section) at 37°C overnight. Use 100 μL solution/section
9. Air dry the sections
10. Assemble the reagents shown in **Table 3**, as found in the EZ-Kit (Perkin-Elmer)
11. Thermocycle at 42°C for 5 min; 65°C for 1h and then 25 cycles of 94°C for 1 min, 58°C for 2 min.
12. Rinse the slides for 5 min in absolute alcohol.
13. To perform ISH, hybridize the section in a moist Terasaki plate at 42°C overnight (10 μL hybridization-mix/section: 7 μL genomic probe mix, 2 μL dsDNA, which is denatured for 10 min in boiling water [approx 50 ng], 1 μL TE)
14. Posthybridization wash with 2X SSC at RT for 15 min is carried out.
15. Incubate the slides for 10 min in TBT
16. Incubate the slides with anti-DIG conjugated peroxidase 1:50 diluted in TBT for 30 min.
17. Rinse in TBS for 5 min (repeated once).
18. Incubate the slides in a freshly made AEC solution for at least 10 min

3.3.5. Detection of Signal

Detection of DIG with alkaline phosphatase conjugated anti-DIG antibody system and NBT/BCIP as for PCR-ISH.

4. Notes

1. Alternatively, the aminoethylcarbazole substrate kit from Zymed (San Francisco, CA) can be used.
2. These solutions have to be made up in DEPC-treated water.
3. The slides can be stored at room temperature until needed. Alternatively, precoated slides are available from Perkin-Elmer precoated with APES.
4. Alternatively, the cells may be fixed in NBF
5. The "crosslinking" fixatives such as formalin or paraformaldehyde allow reagent penetration only after considerable pretreatment of the tissue sections. However, despite the harshness of these pretreatments, the use of these fixatives favors complete preservation of morphology and therefore retention of signal.
6. This step prevents loss of sample during the pretreatment steps.
7. Incubation in detergent solutions produces sufficient permeabilization of fresh culture cells and cell smears.
8. A brief incubation in acetic acid can destroy endogenous alkaline phosphatase activity This is effective for the removal of liver type alkaline phosphatase, but usually is less effective in destroying endogenous intestinal and placental phosphatases.

Table 3
Reagents for In-Cell Amplification with *rTth* Polymerase

Volume added, µL	Reagent	Final concentration
1.85	10 m*M* dATP	370 µ*M*
1.85	10 m*M* dCTP	370 µ*M*
1.85	10 m*M* dGTP	370 µ*M*
1 85	10 µ*M* dTTP	370 µ*M*
10	5X Buffer	1X
8–11	25 m*M* Manganese acetate	4–5.5 mM, best: 5.5 m*M*
5	10 µ*M* Primer 1	1 µ*M*
5	10 µ*M* Primer 2	1 µ*M*
2	2 5 U/µL r*Tth* Polymerase	
Add to 50	DEPC water	

9. This abolishes the endogenous peroxidase activity.
10. The pretreatment of formalin-fixed paraffin sections is described and includes a dilute hydrochloric acid hydrolysis step. With mild acid hydrolysis, some limited depurination of nucleic acids occurs. The hydrochloric acid may also partially solubilize the highly crosslinked acidic nuclear proteins, found in fixed tissues, and allows easier access of PCR reagents.
11. An incubation with detergent solution alone is insufficient for complete permeabilization of cells. For effective permeabilisation, incubation with a protease is essential. Proteinase K is the most effective proteinase for performing this task, without destroying overall cellular architecture The concentration of proteinase K used is critical, if morphology is to be preserved, and is clearly tissue dependent. The optimum concentration for a particular tissue must be determined empirically by titration, using a positive control for hybridization. Pepsin/HCl digestion can also be used (9.5 mL dH_2O, 0.5 mL 2 0 *N* HCl containing 20 mg Pepsin, incubating for 10–30 min at 37°C).
12. For IS-PCR with labeled probes, do not allow the slide to cool to 4°C, as this will lead to spurious binding of labeled primer to cells and tissues. Ideally, the temperature should only be allowed to cool to 5°C below the predicted T_m of the primer.
13 For 5'-labeling, oligonucleotides can be synthesized with an aliphatic amino group that is free for conjugation with several different fluorochromes. Alternatively, alkaline phosphatase can be coupled directly to short synthetic oligonucleotides. These have single modified bases containing a reactive amino groups that enable covalent crosslinking
14. Biotinylated- and DIG- or fluorescent labeled genomic DNA probes are prepared usually at a concentration of 200 ng/mL in one of the hybridization buffers described in **Subheading 2.1.7.** For oligonucleotide probes, concentrations of 5–10 pmol/100 µL are advised. The concentration depends on the particular type of oligonucleotide labeling method employed. When using "tailed" probes, lower concentrations are required.

15 It is usually preferable to use as simple a hybridization buffer as possible and this is usually adequate for producing successful hybridization at temperature ranges of 37–42°C with overnight incubation. Hybridization buffer 2 is an example of this The milk powder present in this buffer successfully blocks nonspecific probe binding sites, which obviates the need for complex mixtures containing phenol, BSA, and polyvinylpyrrolidone. In addition, there appears to be no significant advantage in incorporating a carrier DNA, such as single-stranded DNA (e.g., salmon sperm DNA) into hybridization mixtures For oligonucleotide probes, hybridization buffer 4 is suitable for most applications

16. Hybridization buffers that contain dextran sulfate have a high viscosity. This usually produces a high surface tension on contact with a glass surface Therefore, if glass coverslips are used to cover tissue sections during hybridization reactions, they are difficult subsequently to remove as the surface tension that is induced between the glass and the buffer produces enough suction to lift the section from the slide. For this reason, we recommend the use of gel bond film (FMC), cut to coverslip size to cover the sections. This material is a pliable plastic and is therefore easily removable following hybridization. It also has a hydrophilic and hydrophobic surface. The hydrophobic surface is usually faced downwards toward the tissue section that results in no surface tension with the buffer. Gel bond is sealed in place with nail varnish to prevent the outward leakage of the probe and inward leakage of moisture during hybridization Alternatively, rubber cement or agarose can be used.

17 This step is for denaturing both cellular DNA and probe DNA (if double stranded) Temperature and timing of this step is critical, if tissue morphology is to be preserved

18 For genomic probes, incubation of the slides at 37–42°C overnight results in high hybridization efficiency, particularly with DNA virus probes and is stringent enough to produce specific hybridization with little or no cross-hybridization between viral types For oligonucleotide probes, conditions of hybridization are in general similar to genomic probes Ideally, the T_m of the probe should be calculated. However, classical T_m solution hybridization kinetics do not follow hybridization kinetics within tissue sections

19 The objective of the washing protocol is to remove excess or nonspecifically bound probe and to melt away any mismatched hybrids that may have formed during hybridization To achieve this, washing protocols of increasing stringency (decreasing salt concentration and increasing temperature) are needed

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13

Sequencing of PCR Products

Paul Moss and Swee Lay Thein

1. Introduction

It is often necessary to obtain DNA sequence information following successful amplification. Although polymerase chain reaction (PCR) products may be cloned into a vector and sequenced at a later stage, it is often preferable to sequence PCR products directly, without subcloning, and it is this approach that is addressed in this chapter.

Two main methods for the direct sequencing of PCR products have been developed. The first depends on the separation of the two complementary DNA sequences prior to conventional dideoxy sequencing *(1–3)*. Initially sodium hydroxide was used to dissociate the DNA, but this method proved troublesome because of rapid reannealing of PCR products. A system using a single biotinylated oligonucleotide and streptavidin-coated beads has greatly improved this approach (*see* **Subheadings 2.1.** and **3.1.**).

More recently sequencing reagents have themselves been incorporated into PCR reactions with the advent of "cycle sequencing." A reliable example of this type of reaction is described in **Subheadings 2.2.** and **3.2.**

2. Materials

2.1. Direct Sequencing of PCR Products Using Streptavidin-Coated Beads

1. Oligonucleotides for PCR, one of which should be biotinylated at the 5' end (*see* **Note 1**).
2. Standard buffers for PCR reaction.
3. *Taq* polymerase.
4 Dynabeads® M-280 Streptavidin (Dynal). This should be stored at 4°C and is stable for several months.

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5 TES: 10 m*M* Tris-HCl, pH 8, 1 m*M* EDTA, 100 m*M* NaCl
6 Dynal MPC-E magnet.
7. 0.15 *M* NaOH. This must be freshly prepared. Make up 1 *M* NaOH, and then prepare 0 15 *M* dilution.
8. T_7 DNA polymerase.
9. Reagents for sequencing by dideoxy termination (*see* **Note 2**).
10. Sequencing stop solution (Amersham).
11. 3 *M* Sodium acetate
12. Ethanol.

2.2. Cycle Sequencing

1. Thermo Sequenase cycle sequencing kit (Amersham)
2 Agarose.
3. Gene Clean or Qiagen gel purification kit.
4. Glycerol tolerant gel buffer (Amersham).

3. Methods

3.1. Direct Sequencing of PCR Products Using Streptavidin-Coated Beads

1. Use template DNA in a 100 µL PCR reaction to amplify the target sequence. Include 1.0 µg of genomic DNA or 10 ng of a cloned product, 5 pmol of each oligonucleotide, $MgCl_2$ (*see* **Note 3**), PCR buffer (KCl or NH_4Ac based), *Taq* polymerase.
2. Amplify for 30 cycles, optimizing the annealing temperature to generate a specific product.
3. Remove PCR reaction from under mineral oil.
4. Check amplification by running out 5 µL on a 1% agarose gel
5. Wash 25 µL of Dynabeads twice with 100 µL TES using the MPC-E magnet.
6. Add beads to the PCR products and leave at room temperature for 5 min with occasional agitation.
7. Apply the tube to the magnet and remove the supernatant.
8. Add 100 µL of NaOH 0 15 *M* to the beads and stand at room temperature for 5 min.
9. Remove but do not discard the supernatant that contains the nonbiotinylated DNA strand. This single-stranded template can also be utilized if desired. Precipitate by adding 1/10th volume of 3 *M* sodium acetate, pH 5.6, 300 µL 100% ethanol, and spinning for 15 min.
10. Wash the precipitated pellet with 80% ethanol, dry under vacuum or air, and resuspend in 10 µL water.
11. The biotinylated ssDNA that is attached to the Dynabeads is washed with 100 µL TES, then 100 µL water, and finally resuspended in 20 µL water
12. For sequencing take 5 µL of either ssDNA and 1 pmol of oligonucleotide primer (*see* **Note 4**).
13 Sequencing is performed using the dideoxy termination method.
14 If the biotinylated ssDNA is used as the sequencing template, the new DNA strands generated during sequencing should be eluted off the beads by heating to

65°C for 5 min after the stop solution has been added. After collection of the supernatant, 2.5 µL will give an adequate signal on a 6% denaturing polyacrylamide gel

15. According to the length of the sequence to be read, the gel should be run for up to 2 h before exposure to photographic film overnight.

3.2. Cycle Sequencing

In this method, sequencing reagents are incorporated in the PCR. Basically three approaches can be utilized to label the newly synthesized product: internal labeling using [α-^{35}S]-, [α-^{33}P]-, or [α-^{32}P]-labeled deoxyadenosine triphosphate (dATP) or deoxycytidine triphosphate (dCTP); [γ-^{33}P]- or [γ-^{32}P]-labeled primer and ^{33}P-radiolabeled terminators. ^{35}S-labeled nucleotides are known to give rise to small amounts of volatile radioactive impurities that are accentuated by exposure to high temperatures over long periods during thermal cycling. This method is therefore no longer recommended. α-^{33}P is the preferred radioactive label. The internal labeling protocol involves two steps—first the labeling step and second, the chain-termination step. In radiolabeled primer cycle sequencing, the first step involves end-labeling one of the primers. By far the more superior method involves radiolabeled terminators and Thermo sequenase (Amersham) that is provided in a kit marketed by Amersham.

1. After PCR, check amplification by running 5 µL of a 100-µL PCR on agarose minigel
2. Remove excess primers and dNTPs that can interfere with the sequencing reaction (*see* **Note 5**). The cleanest template is prepared by gel purification Run all of the PCR product in a 1% prep gel, cut the fragment out under UV, and elute DNA using either Gene Clean or Qiagen. After isolation of the DNA fragment, check product by running a small aliquot (2/40 µL) in a 1% agarose minigel
3 For each sequencing reaction prepare four termination mixes (G, A, T, C) using 2.0 µL of the termination master mix and 0.5 µL of [α-^{33}P]ddGTP, [α-^{33}P]ddATP, [α-^{33}P] ddTTP, and [α-^{33}P]ddCTP, each in a separate tube.
4. Prepare the reaction mix by adding 2 µL of reaction buffer, 50–500 ng of DNA, 1 pmol H_2O to make up to 20 µL final volume and 2 µL Thermo sequenase polymerase.
5. Add 4.5 µL of the reaction mix to each of the termination mix, G, A, T, C. Top with a drop of mineral oil.
6. Cycle 30–60 times in the PCR program. A typical program would be 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s (*see* **Note 6**).
7. After cycling, add 4 µL of STOP solution. Remove the mineral oil.
8. Heat samples to 80°C for 2 min and load 2.5 µL in each gel lane (*see* **Notes 7** and **8**).

4. Notes

1. Oligonucleotides purchased from commercial sources can usually be obtained with a biotinylated 5' end at a small extra charge. If oligonucleotides are synthesized "in-house" an amino-modifier is coupled in the final step of conventional cyanoethyl phosphoramide synthesis. A commercial biotin-NHS ester can then

be used to generate the biotin group. Alternatively biotinylated phosphoramidite can be used to synthesize the 5'-biotinylated oligonucleotide directly

2. Dideoxy- and deoxynucleotides can be purchased as stock solutions from which dilutions can be made and kept at –20°C for several months. Alternatively, suitable kits for dideoxy sequencing are available that include deoxyribonucleoside triphosphates (dNTPs), ddNTPs, dithiothreitol (DTT), and formamide solution
3. Initially use $MgCl_2$ at 1.5 m*M* final concentration but this may need modification to optimize the reaction.
4. The nonbiotinylated primer or an internal primer may be used for sequencing.
5. As in all sequencing reactions, purity of DNA is paramount to obtaining a good sequence. Various methods can be used to isolate the PCR product away from the excess primers and dNTPs. This includes treating the PCR product with a combination of exonuclease I and shrimp alkaline phosphatase Alternatively, if one of the primers is biotinylated, Dynabeads can be used to purify the product (*see* **Subheadings 2.1.** and **3.1.**)
6. The annealing temperature in the cycling reaction is based on the length and complexity of the sequencing primer. A guideline is 5°C below the T_d of the primer that is the sum total of G-Cs (4°C each) and A-Ts (2°C each).
7. Although Tris borate can be used as the buffer in polyacrylamide gel electrophoresis, Amersham recommends a glycerol tolerant buffer in view of the high glycerol concentration in the Thermo sequenase polymerase. This gives sharper and better resolution of the bands.
8. Weak signals can be improved by heating the samples in open vials to promote evaporation of water from the formamide reaction mix. Avoid complete evaporation to dryness

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Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Rajko Kusec

1. Introduction

Reverse transcriptase polymerase chain reaction (RT-PCR) has become a well-established and powerful molecular technique for studying ribonucleic acids. It is used in medical diagnostics for detecting viral RNA, in hematology and oncology for detecting chimeric transcripts of rearranged genes *(1)*, and in the broad area of research applications in gene expression studies. Since its introduction *(2,3)* the method is constantly finding new applications, especially in projects aimed at detecting and cloning differentially expressed genes *(4,5)*.

RT-PCR is simple, yet highly reproducible, specific, and sensitive In this chapter, commonly used protocols for performing RT-PCR are described. These are described under the headings of RNA extraction, reverse transcription, and PCR. Recent improvements in the RT-PCR technique are also discussed.

2. Materials

2.1. RNA Extraction

1. Blood cells separation systems:
   a. Mononuclear cell isolation· Histopaque®-1077 (Sigma, Poole, UK).
   b. Mononuclear and polymorphonuclear cell isolation: Polymorphprep® (Nycomed, Oslo, Norway).
2. Commercial RNA extraction kits:
   a. RNA extraction from separated cells: RNAZOL™B (Ambion, supplied by AMS Biotechnology, cat. no CS-104).
   b. RNA extraction from whole blood/marrow: Fastube™ (Vienna Lab, Austria, cat no 2-025), Purescript™ (Gentra USA [distributed by Flowgen UK], cat. no. R5500), Nucleon™ RNA (Scotlab, UK), Messagemaker™ (for polyA+ RNA extraction) (R&D Systems, UK).

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3. Diethyl pyrocarbonate (DEPC)-treated H_2O. Put 1 mL of DEPC (**caution: toxic, work in the fume hood!**) to 1 L of distilled H_2O Mix by gently inverting the bottle. Leave at 37°C overnight and autoclave next day. DEPC inactivates RNAses.

2.2. Reverse Transcription

1. 5X First-strand buffer (Gibco BRL, Gaithersburg, MD). 250 m*M* Tris-HCl, pH 8 3, 375 m*M* KCl, 15 m*M* $MgCl_2$.
2. 0.1 *M* Dithiothreitol (DTT).
3. SUPERSCRIPT™ II RT (Gibco BRL, Cat No. 18064-014)
4. Deoxyribonucleoside triphosphates (dNTPs): mixture of deoxyadenoside triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), each 10 m*M* (Promega, Madison, WI)
5. Hexamers (random primers) (Gibco BRL, cat. no 48190-011)
6. RNAsin® Ribonuclease inhibitor (Promega, cat no. N2511).

2.3. PCR

1. 10X NH_4 reaction buffer (Bioline, UK).
2. 50 m*M* $MgCl_2$ (Bioline)
3. Bio*Taq*™ Polymerase (Bioline).
4. dNTPs: mixture of dATP, dCTP, dGTP, and dTTP, each 10 m*M* (Promega).
5. AmpliWax™ PCR Gem 100 (paraffin beads, Perkin-Elmer, Norwalk, CT, cat no. N8080106).
6. Oligonucleotide primers.
   a. SPARC gene (cDNA):
      i Forward primer (nt139–162): 5'-ACA GAG GTG GTG GAA GAA ACT GTG-3'
      ii Reverse primer (nt460–439): 5'-TGC CCT CCA GGG TGC ACT TTGT-3'.
   b. RARalpha gene (cDNA):
      i. Forward primer (nt235–255): 5'-CAG CAC CAG CTT CCA GTT AG-3'.
      ii. Reverse primer (nt404–388)· 5'-CCA TAG TGG TAG CCT G-3'.

3. Methods

3.1. Extraction of RNA

Any type of RNA (mRNA, tRNA, rRNA, total RNA, viruses), even those present at low copy numbers (e.g., 1–10 copies/cell) can be analyzed. It is important to note that RNA is less stable and very prone to degradation by ribonucleases from tissues and cells. RNAses are also present in the environment so that appropriate precautions (wearing and changing gloves, working on ice, RNase-free plasticware and glassware) must be taken.

The best sources for good quality (undegraded) and quantity of RNA are fresh tissues or cells. If adequately stored (best in liquid nitrogen) RNA

extracted from tissues or cells will be equally suitable for subsequent analysis (*see* **Note 1**). The most frequently used types of RNA for PCR amplification are total (totRNA) or messenger RNA (polyA+RNA).

Preparation and application of RNA from processed, archival, paraffin-embedded material or cytological preparations has been described and is increasingly used ***(6,7)***. It is easier and more reliable to obtain the RNA from a sample of adequate size but it is possible to perform even a single-cell RT-PCR ***(8)***.

1. Homogenize cells/tissue completely in RNAZOL™ B (we use up to 1×10^7 cells with 1.8 mL RNAZOL B in 2 mL Eppendorf tube) (*see* **Note 2**).
2. Add 0.1 volume chloroform to 1 vol of homogenate (e.g., 160 µL of chloroform to 1.6 mL of total volume)
3. Vortex for 20 s, incubate on ice for 15 min and spin at maximum speed (13,000 rpm) in the precooled (4°C) microcentrifuge for 15 min.
4. Transfer the supernatant to a new 1.5-mL Eppendorf tube and precipitate with an equal volume of isopropanol (approx 600–700 µL usually). Precipitation can be as short as 15 min at 4°C. However, we prefer at least 2 h or, for convenience, overnight at –20°C.
5. Pellet RNA for 15 min at maximum speed in a cold microcentrifuge and wash the pellet twice with cold 75% ethanol. Spins with ethanol are shorter· 10 min at 10000g. Mix pellets with ethanol by vortexing.
6 Dry RNA pellets briefly under vacuum or carefully remove ethanol with a fine pipet tip (*see* **Note 3**). Air dry, but do not overdry the pellet.
7. Dissolve RNA in DEPC water: The amount of the water is proportional to the pellet size (usually between 20 and 50 µL) (*see* **Notes 4–6**). **Fig. 1.**
8 Store at –70°C until use.

3.2. Reverse Transcription

Reverse transcription can be performed as a separate reaction from PCR or can be coupled to PCR in the same tube (single tube RT-PCR) by using the *Tth* DNA Polymerase (*Thermus thermophilus*) that acts both as a thermostable RT and a thermostable DNA polymerase in second-strand synthesis and subsequent amplification (*see* **Note 7**). Separating the reverse transcription and amplification steps has the advantage of producing a stock of cDNA that can be used for multiple PCR assays.

The basic elements of the RT reaction are: RNA template, RT, and primers to initiate the transcription to complementary DNA strand.

1. Add the following components to a sterile, nuclease-free, 0.5 mL thin-walled microcentrifuge tube: 4 µL 5X RT buffer, 2 µL 10 m*M* dNTPs, 1 µL Random hexamers (3 µg/µL) (*see* **Note 8**), 2 µL 0 1 *M* DTT, 1 µL RNAsin (40 U/µL), 1 µL Moloney murine leukemia virus (MMuLV) RT (200 U/µL), and 9 µL RNA in DEPC H_2O for a total of 20 µL.
2. Prepare a master mix if more than one reaction is needed.

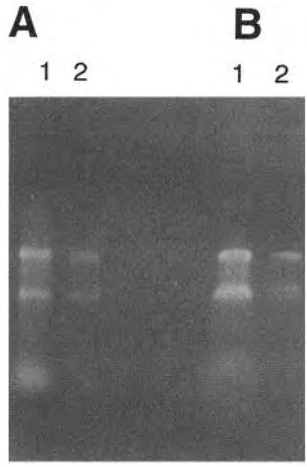

Fig. 1. Testing of total RNA on a standard 1.5% TBE agarose gel. (**A**) Native RNA: lane 1, 1 μg; lane 2, 0.1 μg. (**B**) Sodium dodecyl sulfate (SDS)-denatured RNA: lane 1, 1 μg; lane 2, 0.1 μg. Note slightly stronger signals in denatured samples. Upper band is 28S rRNA (4.9 KB), lower band is 18S rRNA (1.9 Kb). Faint DNA is visible just above 28S.

3. Heat the RNA sample at 65°C for 5 min and cool on ice if pronounced secondary structures are expected (optional).
4. Perform reverse transcription reaction in a thermocycler at 37°C for 60 min, and 65°C 10 min (*see* **Note 8**).
5. Store cDNA at –20°C until DNA amplification.

Although the size and integrity of the first-strand cDNA can be checked by denaturing it with NaOH and running on an agarose gel, the best and most important confirmation of the successful cDNA synthesis and its quality is PCR amplification of the chosen sequence of the gene known to be present in the sample material (a control gene amplification).

3.3. Amplification of cDNA by PCR (see Notes 9 and 10)

This is basically not different from amplification of DNA templates. All the general considerations for successful PCR apply to RT-PCR as well. As an example of an RT-PCR protocol, amplification of the extracellular matrix-associated protein gene SPARC (Secreted Protein Acidic and Rich in Cysteine, Osteonectin) is described:

1. Set up PCR reaction mixture for SPARC gene as shown in **Table 1**.
2. Prepare lower and upper mixture as master mixes.
3. Aliquot first the lower mix into the PCR tubes.

Table 1
Composition of PCR Mix for Amplification of SPARC Gene

Mix component	Volume	Final concentration
Lower PCR		
dNTP mix, 10 m*M* each deoxynucleotide	4 µL	0.2 m*M*
50 µ*M* Forward primer	1 µL	1 µ*M*
50 µ*M* Reverse primer	1 µL	1 µ*M*
25 m*M* $MgCl_2$	3 µL	1.5 m*M*
ddH_20	14 µL	
Total	23 µL	
Upper PCR		
10X reaction buffer	5 µL	1X
Taq Polymerase	0.5 µL	2 5 U/reaction
ddH_20	20.5 µL	
cDNA	1 µL	
Total	27 µL	

Table 2
Cycling Profile for Amplification of SPARC Gene

Step	Temperature	Time
Initial denaturation	94°C	5 min
Step 1: Denaturation	94°C	30 s
Step 2: Annealing	60°C	30 s
Step 3: Elongation	72°C	30 s[a]
Step 4: Final elongation	72°C	5 min
End		

[a]Steps 1–3 for 30 cycles

4. Add in each tube one Ampliwax bead (*see* **Note 11**).
5. Close the tubes and heat them in a thermocycler to 80°C for 5 min.
6. During that incubation prepare the upper mix.
7. Take tubes out of the hot block. Wax solidifies quickly (the process can be sped up by placing on ice).
8. Add an aliquot of the upper mix to each tube
9. Add template (cDNA) last.
10. Spin briefly and start amplification cycles. Cycling conditions are shown in **Table 2**.

The control gene amplified from the same templates is retinoic acid receptor alpha (RARalpha) gene (*see* **Note 12**). The composition of the PCR mixes is

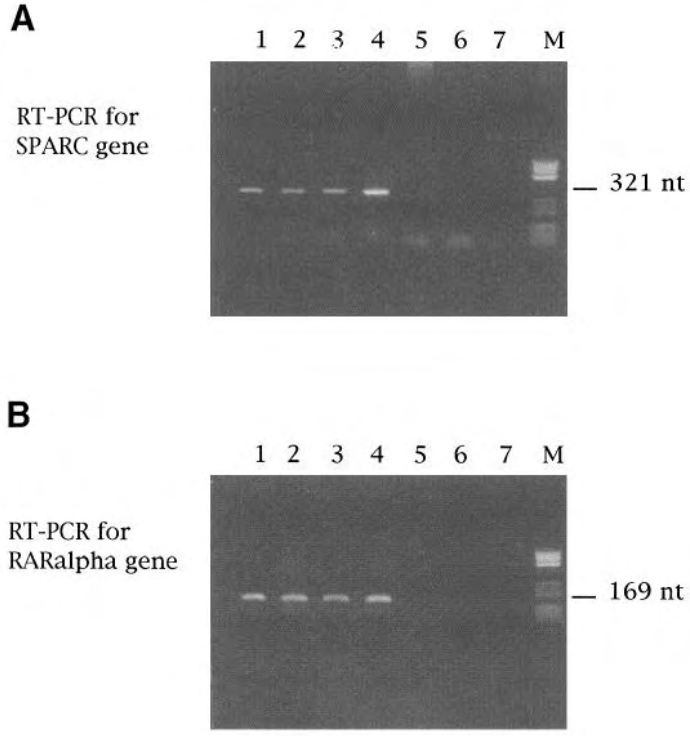

Fig. 2 (**A**,**B**). Lane 1, Peripheral blood mononuclear cells; lane 2, normal bone marrow; lane 3, multiple myeloma bone marrow; lane 4, multiple myeloma cell line Thiel; lane 5, DNA from material in lane 2—bone marrow (note: no amplification); lane 6, negative control for reverse transcription, no RNA in RT; lane 7, no cDNA template (negative control).

the same as for the SPARC gene. Results are shown in **Fig. 2.** The cycling conditions for RARalpha gene shown in **Table 3**.

4. Notes

1. If cells or tissues from which RNA is to be extracted cannot be processed immediately and/or if there are no proper facilities or time for following a special freezing protocol (mixing of materials with 10% dimethyl sulfoxide (DMSO)-media and computer-controlled gradual freezing) and storing in liquid nitrogen, a "snap" freeze should be tried that should sufficiently preserve RNA. Briefly dip the sample in liquid nitrogen to freeze and store it at –70°C.
2. Apart from the RNAZOL B method there are other efficient RNA extraction protocols available. Widely used is the one using cationic surfactant Catrimox-14® for lysis and precipitation of RNA and extraction with guanidinium isothiocyanate ***(9)***. RNAZOL B is a modified guanidinium isothyocyanate, chlo-

Table 3
Cycling Conditions for the RAR Alpha Gene

Step	Temperature	Time
Initial denaturation	94°C	5 min
Step 1: Denaturation	94°C	30 s
Step 2: Annealing	58°C	50 s
Step 3: Elongation	72°C	60 s[a]
Step 4: Denaturation	94°C	30 s
Step 5: Annealing	58°C	30 s
Step 6. Elongation	72°C	30 s[b]
Step 7: Final elongation	72°C	5 min
End		

[a]Steps 1–3 for 10 cycles
[b]Steps 4–6 for 20 cycles

roform-ethanol extraction procedure introduced by Chomczynski and Sacchi ***(10)*** that utilizes the RNA-binding properties of guanidinium isothyocyanate and RNase inhibition.

a. Put 0 8 mL RNAZOL B to the cells Mix and homogenize the cells, then add the other half of the volume. Putting the entire volume at once would cause spilling out of the tube during mixing.
b. Collect the aqueous upper phase while taking care not to disturb the interface and the lower phase, where the DNA and proteins remain. It is preferable to recover less of the aqueous phase if there is any risk of contaminating it with the interface and the lower phase.
c In practice overnight precipitation results in somewhat better RNA yield

3. It is important not to overdry the pellet, which will not dissolve properly, but it is also important to remove the ethanol completely as it is an inhibitor of PCR.
4. RNAZOL B extracted totRNA is of good quality and purity (as measured by the OD260/280 ratio). However, small amounts of DNA may still be present in the preparation (*see* **Fig. 1**)
5. We are mostly extracting RNA from blood cells, obtained after density gradient separation of whole blood (Histopaque® and modified methods e.g., Polymorphprep®). Yields of 10–50 μg RNA are usually obtained from 1×10^7 blood mononuclear cells, but yields from neutrophils are less (can be only 1/10 of mononuclear cell yield) RNA can also be extracted from whole blood and bone marrow, with no previous cell separations. In this situation, red cell lysis is usually performed as the first step. If RNAZOL B is used then the sample/solution ratio should be 1.10. There are also other convenient commercial kits available (examples listed in **Subheading 2.2.**).
6 It is possible to check the RNA quality by running an aliquot on the agarose gel. Standard methods use either denaturing formamide/formaldehyde gel or glyoxal/

DMSO sample denaturation *(11)*. However, running RNA on standard TBE gel will also give sufficient information on RNA quality Prior to loading, samples can be denatured with 1 μL of 20% SDS per 1 μg of RNA at 65°C for 5 min, but as can be seen in **Fig. 1**, the nondenatured sample produced similar signal in the gel. The lower RNA detection limit of agarose gel is 0.1–1 μg

7 For reverse transcription of the RNA molecule into the first cDNA strand there are several choice of enzymes. The first group consists of viral reverse transcriptases (e.g., from the avian myeloblastosis virus [AMV] or from MMuLV). Although these enzymes have similar synthesis processive performance, they may have different optimal reaction conditions (pH, salt concentration, temperature) A different group of enzymes is represented by the *Tth* DNA polymerase which is used for combined reverse transcription and PCR It efficiently reverse transcribes RNA at 70°C that is beneficial for minimizing secondary RNA structures. It has no RNase-H activity (the degradation activity of RNA/DNA hybrids). However, for optimal activity it requires manganese ions that are also damaging to RNA and have to be chelated in the subsequent cDNA amplification step. MMuLV produces long cDNA templates, and has less RNase-H activity. A new, improved, genetically engineered MMuLV RT, e.g., Expand™ reverse transcriptase (Boehringer Mannheim) is also available and long fragments (14 kb) have been successfully reverse transcribed and amplified *(12)*. The SuperScript II™ RT (Gibco BRL) used in the example in this chapter is one of the newer commercially available RT enzymes.

8. For priming of the reverse transcription, a number of primers can be used·
   a. Oligo(dT) 12–18 primer (binds to polyA+ tail), producing full length cDNA,
   b. Random hexanucleotides (hexamers): these randomly bind at complementary sites in the RNA molecule and give partial length cDNAs and they may exhibit preferential transcription of the 5' regions in the template; and
   c. Specific template primer: for selective transcription of the RNA of interest.

   It is interesting to note that it has been shown that RT starting from total cellular RNA leads to cDNA synthesis independent of the addition of primers and that priming by random hexanucleotides may simply be priming by cellular RNA fragments present in the RNA preparations *(13)*. Optimally 1 μg of total RNA or 50–100 ng of PolyA+ RNA should be used in reverse transcription. In practice even without precise measurement of the RNA concentration, volumes of 2–5 μL of stock totRNA can be successfully reverse transcribed. Some protocols also recommend final treatment of the RT reaction with 3–5 min incubation at 95°C to inactivate RT that could interfere with the subsequent PCR.
9. RT-PCR is a method that detects the expression of a gene, not its presence in the genome. It is important therefore to ensure that PCR is amplifying the cDNA and not genomic DNA molecules. Frequently RNA preparations made using the acid guanidinium-isothiocyanate method *(10)* contain DNA. An important test for the cDNA-specificity of the RT-PCR assay is to include a control in which the "reverse transcription" step has been done in the absence of the RT There should be no amplification using this control. Primers for cDNA amplification should

ideally be complementary to different exons, if possible. Because many introns are too long to be amplified by standard PCR systems, the amplification will be cDNA-specific. However, sometimes the ability of the same primers to amplify the cDNA and DNA templates can be deliberately exploited as in the identification of smaller introns by parallel amplification of both types of template and comparison of the amplified products ***(14)***.

10. The control of the possible carryover contamination should be appropriate. For the RT-PCR assay that means running of the "mock" reverse transcribed sample (water instead of RNA) plus standard PCR no-template negative control (**Fig. 2**). If nested PCR is used, then aliquots from both negative control samples from the first PCR should be taken into the next PCR step.
11. It has been shown that separating enzyme from primers during the initial preparation of PCR mixture and before the first denaturing temperature significantly increases specificity and sensitivity of the assay The simple solution suggested is to prepare two PCR master mixes: one with primers, dNTPs; and template and the second one with buffer, Mg, and polymerase. Both parts should be aliquoted and mixed just immediately before cycling ***(12)***. In a genuine "hot-start PCR," usually the enzyme is witheld until the reaction reaches first denaturing temperature and is then added manually. We find the use of wax barrier (wax beads, AmpliWax) practical and efficient The "lower" and "upper" PCR mixes (*see* **Subheading 3.3.**) are separated by a wax layer that melts above 70°C and allows mixing of two parts when reaching the denaturing step. Other "hot-start" modifications use polymerase antibodies with inhibition of polymerase activity being completely reversed when temperature is raised in the first denaturation step ***(15)***
12. Inclusion of amplification of the control gene, a gene known to be present in the materials tested, is important It confirms the specificity of the assay for cDNA amplification and, the abundance of template and its quality. When choosing the control gene assay to accompany the experimental gene amplification, the same rules on the intron/exon structure in the design should be respected. If possible, genes that have no known pseudogenes should be selected. For instance, the very frequently used beta-actin gene has many processed pseudogenes ***(16)*** and is not an ideal RT-PCR control gene ***(17)***. It may also be expressed at a higher level than the gene(s) of interest ***(18)***, as is the case for many other housekeeping genes. To overcome this problem, in the study of chromosomal translocations in cancers, many investigators use one of the unaffected partner genes (e.g., the *bcr* or *abl* gene in *bcr/abl* rearrangement of t[9;22]) as a control gene.

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15

PCR Amplification of Minisatellite DNA for the Detection of Mixed Chimerism After Bone Marrow Transplantation

Etienne Roux

1. Introduction

Allogeneic bone marrow transplantation (BMT) has become a recognized therapy for the treatment of patients with leukemia, severe combined immune deficiency (SCID) and severe aplastic anemia (SAA). It was originally assumed that complete donor chimerism was essential for sustained engraftment. However, despite high dose chemoradiotherapy given as preconditioning, the persistence of host hematopoietic cells (mixed chimerism) has commonly been observed after BMT and the relationships between mixed chimerism, engraftment and relapse have remained controversial *(1–4)*. To evaluate the clinical relevance of mixed chimerism after BMT, we developed a method based on the amplification of minisatellite DNA regions by the polymerase chain reaction (PCR) *(5,6)*.

Minisatellites are composed of a variable number of locus-specific consensus sequences that vary between 14 and 100 bp in length *(7)*. These structures also called variable number of tandem repeats (VNTR) are abundant in the genome and are among the most variable human loci. PCR amplification of these regions make it possible to distinguish the genotypes of recipient (R) and donor (D) in virtually any situation except when the donor is an identical twin. Application of the method involves the identification of informative loci with D- and R-pretransplant DNA. One locus (or more) revealing R- or R- and D-specific fragments is used for post-BMT analysis. The length polymorphism is revealed by agarose gel electrophoresis and ethidium-bromide staining. The sensitivity of the procedure can be increased after transfer of the DNA on membranes and hybridization with allele-specific oligoprobes.

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The description of the method is divided in four parts:

1. Cell separation
2. DNA extraction
3. PCR conditions
4. Analysis of the PCR products.

2. Materials

1. 1X Phosphate-buffered saline (PBS): 0.14 *M* NaCl, 4 m*M* KCl, 9 m*M* phosphate buffer, pH 7.4.
2. Ficoll: Ficoll paque (Pharmacia, Uppsala, Sweden).
3. Lysis buffer: 0.32 *M* sucrose, 10 m*M* Tris-HCl, pH 7.6, 5 m*M* $MgCl_2$, 1% Triton X-100.
4. TN Buffer: 10 m*M* Tris-HCl, pH 7 6, 10 m*M* NaCl
5. Proteinase K: 20 mg/mL in H_2O. Dispense into small aliquots and store at –20°C.
6. 5X PCR buffer. 250 m*M* KCl , 50 m*M* Tris-HCl, pH 8.8 (at 20°C), 5 (7.5) m*M* $MgCl_2$, 500 µg/mL bovine serum albumin (BSA).
7. 2X S1 nuclease buffer: 60 m*M* Na-acetate pH 4.6, 100 m*M* NaCl, 2 m*M* $ZnCl_2$, 20 µg/mL BSA.
8. Loading buffer: 40% sucrose, 1X TBE, 0 1% bromophenol blue, 0.1% xylene cyanol.
9. 0.5 *M* Ethylenediamine tetra-acetic acid (EDTA): adjust the pH to 8 0 with NaOH.
10. 10% Sodium dodecyl sulfate (SDS): 100 g SDS in 900 ml water. Toxic! Wear a mask when weighing SDS.
11. 1X TBE: 89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA.
12. Ethidium bromide: stock solution at 10 mg/mL. Wrap the container in aluminium foil and store at 4°C. **Powerful mutagen!**
13. [^{32}P]Adenosine triphosphate (ATP): hazardous because of its high ß-energy and its elevated specific activity. Use plexiglas screen.
14. 1X T4 kinase buffer: 70 m*M* Tris-HCl, pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol (DTT).
15. 5X Sodium chloride/Sodium phosphate/EDTA (SSPE): 50 m*M* N-phosphate buffer, 0.9 *M* NaCl, 5 m*M* EDTA.
16. 5X Standard saline citrate (SSC): 0 75 *M* NaCl, 0.75 *M* sodium citrate.

3. Methods

3.1. Cell Separation by Ficoll Gradient Centrifugation

To distinguish lymphoid from myeloid chimerism, the analysis is performed on mononuclear cells (MNCs) and granulocytes separately (*see* **Note 1**). The two fractions are separated by Ficoll-Hypaque density gradient centrifugation.

1. Dilute 10 mL of blood with 10–20 mL 1X PBS.
2. In a 50-ml tube, load carefully the diluted blood solution onto 15 mL Ficoll
3. Centrifuge for 10 min at 800*g*
4. Remove the MNC fraction (between the Ficoll and the PBS) and transfer to a second 50-mL tube. Add 1X PBS to 50 mL to wash the MNC fraction.

5 Remove the Ficoll and the PBS from the original tube and add 1X PBS to 50 mL to wash the granulocyte pellet.
6. Centrifuge both fractions (the MNC from **step 4** and the granulocytes from **step 5**) for 10 min at 400*g*.
7 Remove both supernatants The cells are ready for the DNA extraction.

3.2. DNA Extraction

Quick DNA preparations are made at room temperature in 1.5-mL microcentrifuge tubes following a procedure described by Grimberg et al. *(8)* with some modifications.

1. In 1 mL lysis buffer, resuspend 3×10^6 MNC (18 µg DNA) or add 200 µL of the granulocyte suspension.
2. Spin down nuclei at 6700*g* in a microcentrifuge for 1 min
3. Pour off the supernatants and repeat **steps 1** and **2** with the granulocyte pellet.
4. Resuspend the pellets in 1 mL TN buffer.
5. Centrifuge at 6700*g* for 1 min (*see* **Note 2**).
6. Remove the supernatants and resuspend the pellets in 100 µl PLB + 0.5 mg/mL proteinase K (*see* **Note 3**)
7. Incubate for 2 h at 65°C.
8. Denature the proteinase K for 5 min at 95°C.
9. Centrifuge at 6700*g* for 30 s.
10. Store the samples at 4°C (*see* **Note 4**).

3.3. Polymerase Chain Reaction

For the PCR amplifications, we synthesised primers flanking the loci 33.1, 33.6, YNZ 22, Apo B and St 14. Some properties of these minisatellite loci are described in **Table 1**.

3.3.1. PCR Incubation Mixtures

PCR reactions in 0.5-mL microcentrifuge tubes performed in 20 µL containing: 1–1.5 µL DNA solution (200–300 ng genomic DNA), 4 µL 5X PCR buffer, 0.4 µL 10 m*M* deoxyribonucleoside triphosphates (dNTPs) (0.2 m*M* final), 0.4 µL each primer 100 ng/µL (6 pmol), 0.5 U *Taq* DNA polymerase (Boehringer Mannheim), H_2O to 20 µL. The mixtures are overlaid with paraffin oil. In all experiments, a blank sample (without DNA) is included to monitor for DNA carryover. The author uses the thermal cyclers Techne PHC-1 and PHC-2.

3.3.2. Cycling Parameters

The following cycling conditions are used:

1 33.1: 1 m*M* $MgCl_2$, 94°C for 0.5 min, 65°C for 0.5 min, 72°C for 1.5 min
2. 33.6: 1.25 m*M* $MgCl_2$, 94°C for 0.5 min, 60°C for 0.5 min, 72°C for 1 min.

Table 1
Properties of the Minisatellite Loci and Sequence of Synthetic Oligonucleotides

Locus	Chromosomal location	Allele number	PCR primers (A and B) and probe (P) (5' to 3')	Repeat size	Refs
33.1	9q	10	A: CTTTCTCCACGGATGGGATGCCAC B: GCCGTGTCACCCACAAGCTTCTGG P: GGGTGGGCAGGAAGTGGAG	62	*7,9*
33.6	1q23	8	A: TGTGAGTAGAGGAGACCTCACATT B: AGGTGAGACATTACTCAATCCAAG P: AGGGCTGGAGGAGGGCTGGAGG	37	*7,9*
YNZ 22	17p13	>12	A: CGAAGAGTGAAGTGCACAGG B: CACAGTCTTTATTCTTCAGCG P: CAGAAGCAATGAGGGCTTGAGG	70	***10,11***
St 14	Xq28	10	A: GGCATGTCATCACTTCTCTCATG B: CACCACTGCCCTCACGTCACTT P: TGTGTGTATATGTATATG	60	***12,13***
Apo B	2p24-p23	>12	A: ACGGAGAAATTATGGAGGGA B: CCTTCTCACTTGGCAAATAC P: CGAGGTTATTGATCTCAGG	14	***14***

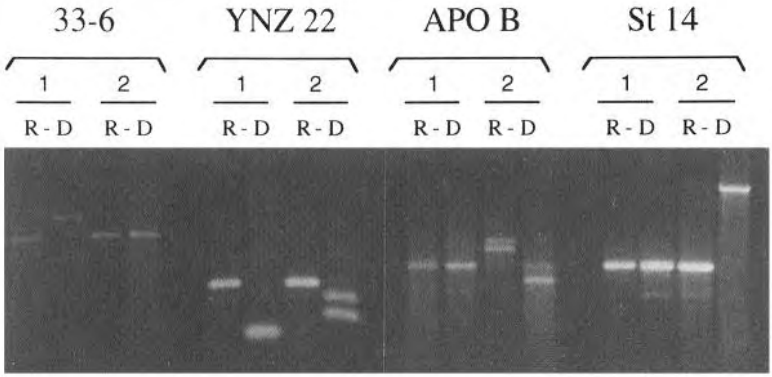

Fig. 1. Screening for informative markers for two R–D pairs. R-specific fragments were found with 33.6 and YNZ 22 primers for pair 1 and with YNZ 22, Apo B and St 14 primers for pair 2. The four primers were analyzed together using the sequential annealing temperature protocol (*see* **Note 5**).

3. YNZ 22: 1 m*M* $MgCl_2$, 94°C for 0.5 min, 60°C for 0.5 min, 72°C for 1 min (*see* **Note 5**).
4. Apo B: 1.25 m*M* $MgCl_2$, 94°C for 0.5 min, 60°C for 0.5 min, 72°C 1 min.
5. St 14: 1 m*M* $MgCl_2$, 94°C for 0.5 min, 60°C for 0.5 min, 72°C for 1 min.

All reactions are initiated by a denaturation step of 3 min at 94°C and are terminated with a final extension of 8 min at 72°C, 30 cycles are performed except for 33.1 (27 cycles). Note that by varying the $MgCl_2$ concentrations, DNA can be amplified in parallel with the primers 33.6, YNZ 22, Apo B, and St 14 using the same cycling parameters (**Fig. 1**). After completion of the amplifications, the samples are stored at –20°C.

The percentages of cases in which informative markers are found for recipient or recipient and donor at each minisatellite are given in **Table 2**. Informative markers specific for the recipient DNA were found in 160/162 (98.8%) R–D pairs.

3.4. Analysis of PCR Products

The amplification products contain a small proportion of single-strand DNA resulting from incomplete extension products which could not prime further *(9)*. During the hybridization, these fragments of heterogenous sizes can also hybridize to the oligoprobe and the resulting background may reduce the sensitivity of the method. Because most of the background can be eliminated by digestion with single strand-specific nuclease (S1, mung bean) a brief nuclease digestion is usually performed prior to the separation on agarose gels *(9)*.

Table 2
Percentage of Cases in which Informative Markers were Identified for Recipient (R) or Recipient and Donor (R+D) at Each Minisatellite Locus

	Sibling donor		Unrelated donor		
Locus	R	R + D	R	R+D	Heterozygosity %
33 1	52/120 (43%)	33/120 (27%)	14/20 (70%)	10/20 (50%)	66
33.6	58/129 (45%)	44/129 (34%)	15/19 (79%)	13/19 (68%)	67
YNZ 22	61/99 (62%)	49/99 (49%)	9/10 (90%)	9/10 (90%)	86
Apo B	38/77 (49%)	31/77 (40%)	9/13 (69%)	8/13 (61%)	75
St 14	5/12 (42%)	5/12 (42%)	—	—	80

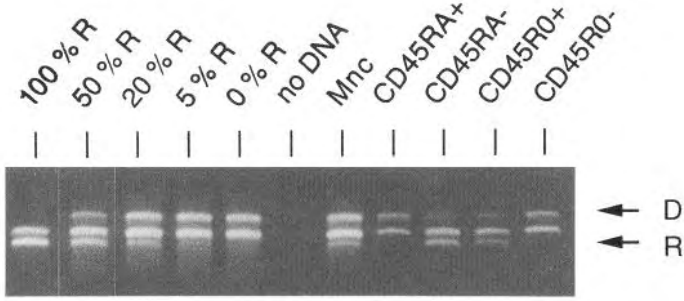

Fig. 2. Detection of mixed chimerism in different cell populations. DNA was extracted from 7000 $CD3^+CD45RA^+$, 33000 $CD3^+CD45RA^-$, 18000 $CD3^+CD45RO^+$ and 31000 $CD3^+CD45R0^-$ cells in 10 µL proteinase K solution. For amplification using the Apo B primers, 1.5 µL was used. Thirty-two cycles were performed. The percentage of host cells is evaluated by comparison with samples of pre-BMT R- and D-DNAs mixed in various proportions (*see* **Notes 6** and **7**). The positions of the R- and D-specific fragments are indicated with arrows.

3.4.1. Agarose Gel Electrophoresis

1. Incubate 10 µL of the amplified sample with an equal volume of 2X S1 nuclease buffer containing 30 U S1 or 2 U mung bean nuclease for 10 min at 37°C.
2. Stop the reaction by adding 2 µL of loading buffer containing 50 m*M* EDTA and 1% SDS.
3. The samples are fractionated on 0.7% (33.1) or 1% agarose gels. The gels are prepared and run in 1X TBE and contain 0.5 µg/mL ethidium-bromide.
4. Run the gel (14 × 20 cm) at 5 V/cm and examine the gel under ultraviolet illumination (**Figs. 1** and **2**).

3.4.2. DNA Transfer by the Alkaline Transfer Method (15)

1. Soak the gel in 0.5 *M* NaOH, 30 m*M* NaCl for 30 min.
2. Wet the Nylon membrane (Nytran, Schleicher & Schuell, Dassel, Germany) in water, then in 0.5 *M* NaOH, 30 m*M* NaCl.
3. Load the membrane on the gel, add two layers of Whatmann 3MM papers and 2 cm of absorbent paper tissues.
4. Transfer for 2–3 h.
5. Wash the membrane in 0.5 *M* Tris-HCl pH 7.6, 30 m*M* NaCl for 15 min.
6. Let dry at room temperature.

3.4.3. Labeling of Oligonucleotides

Radioactive 5'-labeling with [^{32}P]γ-ATP >3000 Ci/mmol and T4 polynucleotide kinase is performed in 10 µL reaction as described ***(16)***.

1. Mix 1 µL oligo 100 ng/ µL (15 pmol), 25 µCi [^{32}P] γ-ATP, 1 µL 10X kinase buffer and 5 U T4 polynucleotide kinase (New England Biolabs).

2. Incubate 30 min at 37°C.
3. Add 10 µL 5% SDS, 10 m*M* EDTA to stop the reaction.

The unincorporated labeled-ATP is not removed from the sample and the mixture is stored at –20°C.

3.4.4. Hybridization and Washing

1. The membrane is prehybridized for 10 min at 55°C in 5X SSPE, 0.1% SDS.
2. Hybridize 3 h at 55°C in 5X SSPE, 0.1% SDS with 10 ng of the labeled probe/50 cm^2 of membrane (= 0.3 pmol labeled oligo/cm^2).
3. The blot is washed twice in 5X SSC, 0.1% SDS for 10 min at 37°C
4. The blot is dried at room temperature and exposed at –70°C with intensifying screens

Time requirement for the total procedure

1. Day 1:
   a Cell separation: 1 h.
   b DNA extraction: 2 h
   c. PCR amplification· 2 h.
   d. Gel electrophoresis. 1–3 h.
   e. Gel transfer: 2 h (or overnight).
2 Day 2:
   a. Blot washing and drying. 1 h.
   b. Hybridization and washing: 3 h.
   c. Exposure: 10 min to a few hours.

4. Notes

1. The relationship between mixed chimerism and graft rejection or relapse is still a matter of debate *(1–4)*. We and others have shown that during remission, mixed chimerism was caused by R–T cells whereas during the relapse, R-cells from other lineages were detected *(3,17)*. The discrimination between the expansion of surviving R-T cells (lymphoid mixed chimerism) and a potential residual activity of the host bone marrow (myeloid mixed chimerism) appears, therefore, important to assess the clinical relevance of mixed chimerism.
2. The nuclear pellets can be stored for months at –20°C.
3. Good results can also be obtained when limited number of cells are available. Adapt the volume of the proteinase K solution to the initial number of cells. Use 1 µL solution for 3X 10^4 cells, and incubate in 10 µL with $<3 \times 10^4$ cells **Figure 2** shows the results obtained after DNA extraction from different numbers of cells ranging from 7000 (CD45 RA^+) to 33,000 cells (CD45 RA^-). Proteinase K digestion is performed in 10 µL and the number of amplification cycles is increased from 30 to 32. DNA fragments are detected by simple ethidium-bromide staining
4. This protocol yields DNA suitable for PCR amplification in the vast majority of cases. When unsatisfactory results are obtained, the purity of the DNA can be improved by phenol/chloroform extraction and ethanol precipitation as follows

a. Add 100 µL phenol (saturated with TN buffer) to 100 µL DNA solution and vortex for 15 s.
b. Centrifuge for 1 min at 6700*g*.
c. Transfer the upper aqueous solution in a second tube and repeat **steps 1** and **2**.
d Add an equal volume of chloroform to the aqueous phase and vortex for 15 s.
e Centrifuge for 1 min at 6700*g*.
f. Transfer the upper aqueous phase in a new tube and add 0.1 vol 0 3 *M* Na-acetate and 2.2 volumes cold (–20°C) ethanol.
g. Mix well and centrifuge for 10 min at 6700*g*.
h. Remove the supernatant and dry the pellet.
i. Resuspend in 100 µL water at 37°C.
j. Take 1–1.5 µL for PCR amplification.

5. The yield of PCR products from the YNZ 22 primers can be improved by using sequential annealing temperatures as follows:
   a. Cycles 1 to 5: 62°C for 60 s.
   b Cycles 6 to 15. 60°C for 70 s.
   c. Cycles 16 to 30· 58°C for 80 s

The same conditions can be applied to the 33.6, Apo B and St 14 primers but they do not improve the yields as compared to the standard protocol.

6. For quantification of mixed chimerism, different ratios of pretransplant R- and D-DNA are amplified and analyzed in parallel with post-BMT samples. This requires the determination of the DNA concentrations of the pre-BMT samples.
7. Because the efficiency of the amplification is reduced for longer fragments, the sensitivity of the method is dependent on the relative sizes of the fragments. Usually, 5–10% of the minor population can be detected after ethidium-bromide staining. The sensitivity is improved to 0.1–1% after hybridization.

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16

Diagnosis of Mitochondrial Disorders Using the PCR

Karl J. Morten

1. Introduction

A large number of mitochondrial disorders have been associated with mutations in mitochondrial DNA (mtDNA) *(1–5)*. Disorders of mtDNA can be divided into three groups: large rearrangements of the mitochondrial genome, point mutations in transfer RNA (tRNA) or coding genes, and a reduction in mtDNA copy number. Only point mutations are currently diagnosed by polymerase chain reaction (PCR) methods. Rearrangements and mtDNA depletion require southern or dot blot analysis. Most pathogenic point mutations described so far can be easily screened using PCR-based methods. Diagnosis of mtDNA disorders is complicated by heteroplasmy, which is unique to this group of diseases. In a normal individual, all of the thousands of copies of mtDNA per cell are identical (homoplasmic). Pathogenic mutations are usually heteroplasmic: a mixture of mutant and wild-type mtDNA molecules coexisting in the same cell or organelle. In many cases the level of mutant in an affected tissue correlates well with disease severity. Ideally, a screening test to detect a pathogenic point mutation should not only identify the presence or absence of a pathogenic mutation, but also quantitate the level of the mutation compared to wild-type mtDNA. Point mutations that result in either a restriction site loss or gain can be identified by amplifying around the mtDNA region of interest and digesting the amplified fragment (e.g., Goto et al. *[2]*). However, the majority of point mutations do not result in the gain or loss of a restriction site. Most of these mutations can be screened by designing a PCR primer containing a series of mismatches which generate a new restriction site in the presence of the mutation *(6)*.

Screening for pathogenic mtDNA point mutations is a relatively straightforward technique with few potential problems. Most of the problems in the diag-

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nosis of mtDNA point mutations are caused by the extreme heterogeneity of the mitochondrial myopathies. Very few pathogenic mtDNA point mutations cause recognized syndromes. Leber's hereditary optic neuropathy (LHON), in which patients present in adolescence with acute loss of vision, is one such syndrome closely linked to pathogenic point mutations in subunits of complex I; at bp 11,778 (G-A) ND4, 3460 (G-A) ND1, and 14,484 (T-C) ND6 ***(4,7–9)***. Myoclonic epilepsy and ragged red fibres (MERRF) and mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) are two well characterized syndromes associated with point mutations at bp 8344 (A–G) in tRNA lysine and bp 3243 (A–G) in tRNA leucine (UUR), respectively. Most patients with the MERRF syndrome have the 8344 (A–G) mutation, but other patients can also have MERRF with a mutation at 8356 (T–C) ***(3,6,10,11,12)***. Variability of clinical presentation with single pathogenic point mutations is shown dramatically with the 3243 (A–G) commonly associated with the MELAS syndrome ***(13,14)***, which has recently been shown to be the likely cause of some cases of diabetes and deafness ***(15,16)***. Other point mutations at bp 3271 and 3252 have also been shown to give either true MELAS or a MELAS-like condition ***(17,18)***. It is, therefore, probably appropriate to initially screen patients with a multisystem disorder for the common mtDNA mutations such as rearrangements and point mutations at 8993, 8344, and 3243. Patients with a particular syndrome can then be screened for the rarer point mutations where few cases, sometimes only one, have been documented. Which patients warrant investigation for mitochondrial disease is outside the scope of this chapter but is reviewed in a recent article by Poulton and Brown ***(19)***.

2. Materials

1. Reticulocyte standard buffer (RSB): 10 m*M* Tris-HCl, pH 7.4, 10m*M* NaCl, 10m*M* ethylenediamine tetra-acetic acid (EDTA).
2. Proteinase K (20 mg/mL).
3. 10% Sodium dodecylsulfate (SDS).
4. Phenol, chloroform, isoamylalcohol
5. 4 *M* NaCl.
6. Hand-held homogenizer
7. 4 *M* NaCl.
8 *Taq* polymerase
9. PCR water (sterile Milli-Q or injection water).
10. 10X PCR buffer: 100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 500 m*M* KCl, 1 mg/mL gelatine, pH 8.3 at 20°C.
11. Nucleoside triphosphate (NTP) mix (5 m*M* of deoxyadenosine triphosphate [dATP], deoxyguanosine triphosphate [dGTP], deoxycytidine triphosphate [dCTP], deoxythymidine triphosphate [dTTP]).
12. PCR primers (*see* **Tables 1** and **2**).

Table 1
Pathogenic Point Mutations Resulting in a Site Gain or Loss

Mutation	PCR primers	Restriction enzyme	Fragment sizes produced when the mutation is present
Site gain			
3243 (A–G)	(F) AGGACAAGAGAAATAAGGCC(3130–3149)	*Apa*I	113 bp and 180 bp
MELAS[a]	(R) GACGTTGGGGCCTTTGCGTA(3423–3404)		
(2)	(Use standard PCR conditions with a 30 s extension)		
8993 (T–G)	(F) GACTAATCACCACCCAAC(8648–8667)	*Ava*I or *Hpa*II	345 bp and 206 bp
NARP[b]	(R) TGTCGTGCAGGTAGAGGCTT(9180–9199)		
(5)	(Use standard PCR conditions)		
8993 (T–C)	(F) As for the 8993(T–G) mutation	*Hpa*II	345 bp and 206 bp
Leighs	(R) As for the 8993(T–G) mutation		
(20)			
11778 (G–A)	(F) CAGCCACATAGCCCTCGTAG (11632–11651)	*Mae*III	146 bp and 84 bp
LHON	(R) GCGAGGTTAGCGAGGCTTGC (11843–11862)		
(4)	(Use standard PCR conditions)		
Site loss			
15257 (G–A)	(F) GCCATGCACTACTCACCAGA(14900–14919)	*Acc*I	Product uncut, 449 bp
LHON	(R) TGCAAGAATAGGAGGTGGAG(15330–15349)		
(21)	(Use standard PCR conditions)		
3460 (G–A)	(F) CAGTCAGAGGTTCAATTCCTC (3275–3295)	*Bsa*HI	product uncut, 302 bp
LHON	(R) TGGGGAGGGGGGTTCATAGTA (3557–3577)		
(7,8)	(Use standard PCR conditions)		
11778 (G–A)	(F) CAGCCACATAGCCCTCGTAG (11632–11651)	*Sfan*I	product uncut, 230 bp
LHON	(R) GCGAGGTTAGCGAGGCTTGC (11843–11862)		
(4)	(Use standard PCR conditions)		
3302 (A–G)	(F) As for the 3243 mutation	*Mse*I	159 bp and 32 bp
Myopathy	(R) As for the 3243 mutation		
(22)	(Use standard PCR conditions)		

[a]Also associated with diabetes and deafness and progressive external ophthalmoplegia (PEO).
[b]Also associated with Leighs disease.

Table 2
Pathogenic Point Mutations Detected by Mismatch Primers

Mutation	PCR primers	Restriction enzyme	Fragment sizes produced when the mutation is present
Site gain			
3271 (T-C)	(F)AGGACAAGAGAAATAAGGCC(3130–3149)	*Bfr*I (*Afl*II)	140 bp and 30 bp
MELAS	(R)TAAGAAGAGGAATTGAACCTCTGAC**CT**TAA		
(17)	(3301–3272)		
	(Use standard PCR conditions except anneal at 45°C for 30 s and extend at 72°C for 2mins)		
8344 (A–G)	(F)GGTATACTACGGTCAATGCTCTG(8155–8176)	*Bgl*I	189 bp and 27 bp
MERRF	(R)GGGCATTTCACTGTAAAG**CC**GTGTTGG (8345–8371)		
(6)	(Use standard PCR conditions Carry out 40 cycles extending for 30 s with a 1 min extension on the last cycle)		
3260 (A–G)	(F)AGGCCTACTTCACAAAGCGCCTTCCC (3145-3170)	*Xmn*I	134 bp and 26 bp
Cardiomyopathy	(R)ATTGAACCTCTGACTGTAAAG**GAA**TAAGTT(3261-3290)		
(23)	(Use standard PCR conditions)		
14484 (T–C)	(F) AGTATATCCAAAGACA**GG**CA(14,464–14483)	*Ban*I	105 bp and 20 bp
LHON	(Morten, unpublished)		
(9)	(R) GGTTTAGTATTGATTGTTAGCG(14,568–14,589)		
	(Use standard PCR conditions except with annealing being performed at 48°C)		

8356 (T–C)	(F)TTTCATGCCCATCGTCCTAG(8200-8219)	*Xba*I	35 bp, 70 bp,
MERRF/MELAS	(R)CATACGGTATTAGTTAGTTGGGGCATTTCACT		and 86 bp
(12)	**C**TA (8357–8391)		
	(Use standard PCR conditions)		
3252 (A–G)	(F) As for 3243 (3130–3149)	*Acc*I	125 bp and 19 bp
Myopathy	(R) TAAAGTTTTAAGTTTTA**GT**CGA (3253–3275)		
(18)	(Use standard PCR conditions		
	with annealing at 48°C and an extension of 30 s)		
11084 (A–G)	(F)CTCTACCTCTCTATACTAATCTCCCTACAAATC	*Nhe*I	243 bp and 44 bp
MELAS	TCCTTAAT**GG**TA(11039–11083)		
(24)	(R)GAGTTTGATAGTTCTTGGGCAGTG(11302–11325)		
	(Use standard PCR conditions except with annealing		
	for 90 s at 56°C and extending for 2 min at 72°C)		
3250 (T–C)	(F)GGTTTGTTAAGATGGCAGAG**G**CCGG(3225–3249)	*Nae*I	177 bp and 22 bp
Myopathy	(R)CACGTTGGGGCCTTTGCGTA (3404–3423)		
(25)	(Use standard PCR conditions except annealing		
	for 30 s at 45°C and extending for 2 min at 72°C)		
Site loss			
3303 (C–T)	(F) GTCAGAGGTTCAATTCCTCTTGTT (3277–3300)	*Hpa*I	Product uncut, 76 bp
Cardiomyopathy	(R) CGATTAGAATGGGTACAATG (3353–3332)		
(20)	(Use standard PCR conditions)		

[a]Mismatch sites are underlined and in bold type

13. ^{32}P dCTP.
14. Mineral oil.
15. Agarose, Nusieve agarose, and acrylamide gel components.
16. TBE: 0.09 *M* Tris-borate, 0.002 *M* EDTA, pH 8.0.
17. Ethidium bromide
18. Restriction enzymes (*see* **Tables 1** and **2**).

3. Methods

3.1. DNA Extraction

3.1.1. Blood

Total DNA is extracted from blood using proteinase K digestion and phenol–chloroform extraction.

3.1.2. Muscle

Total DNA is prepared from muscle biopsies as follows:

1 Grind 10–50 mg of muscle in liquid N_2 using a hand-held homogenizer to produce a fine powder
2. Resuspend sample in 500 µl of RSB buffer, thaw, and homogenize again for an additional 1–2 min.
3. Add 55 µL of 10% SDS and 25 µl proteinase K (20 mg/mL) and incubate overnight at 37°C.
4. Extract samples once with phenol (1 vol·1 vol), once with phenol.chloroform (1.1) 1 vol: 1 vol, and once with chloroform 1 vol:1 vol (*see* **Note 1**).
5. Precipitate DNA with 0 1 vol of 4 *M* NaCl and 2.5 vol ethanol and leave overnight at –20°C.
6 Spin at 13,000 rpm in a microcentrifuge for 20 min and wash pellet with ice-cold 70% ethanol and recentrifuge at 13,000 rpm for 15min
7. Remove ethanol and air or vacuum-dry pellet, resuspend in 50 µL TE buffer. The time taken for complete resuspension depends on the size of pellet and may take up to 24 hr at 4°C.
8 DNA concentrations can be estimated spectrophotometrically at 260 nm or by running on a 0.8% agarose gel against a standard of known concentration

3.2. Standard PCR Conditions (see Notes 2 and 7)

1. Dilute patient DNA samples to give a working stock of approximately 50 ng/µL.
2 Prepare PCR reactions by mixing 2.5 U *Taq* polymerase, 1–1.5 µ*M* PCR primers (**Tables 1** and **2**), 5 µ*M* NTPs, and 5 µL 10X PCR buffer. Make up to a final volume of 49 µL with PCR water and overlay with mineral oil
3. Incubate reactions at 80°C and add 1 µL DNA (50 ng/µL) (hot start)
4. Incubate reactions using the following cycle conditions.

1 Cycle	33 Cycles	1 Cycle
94°C for 5 min	94°C for 1 min	94°C for 1 min

55°C for 1 min	55°C for 1 min	55°C for 1 min
72°C for 1 min	72°C for 1 min	72°C for 2 min

5 Check 5 µL of the PCR reaction on a 1–2% agarose gel If PCR has worked, set up restriction digests as in **Subheading 3.4.**

3.3. Mismatch PCR

Point mutations not resulting in loss or gain of a restriction site are identified by engineering a new restriction site utilizing the point mutation and a series of mismatches in one of the PCR primers (**Fig. 1**). Following amplification, a new restriction site is produced in the presence of the point mutation (**Fig. 1**). Mismatch primers which result in a restriction site loss in the presence of a mutation have also been used successfully *(26)*. mtDNA mutations currently screened by the mismatch PCR primer approach are shown in **Table 2**. PCR conditions for mismatch primer PCR are as in section 3.2 unless stated otherwise (*see* **Table 2**).

3.4. Restriction Enzyme Digestion of PCR Products

This is technically straightforward. However, because low levels of mutant mtDNA may be missed, it is a potentially problematical area, especially if quantitative estimates of mutant levels are required.

1. Carry out PCR reactions in 1X PCR buffer. PCR products with little nonspecific amplification where 5 µL of product can be easily seen on an agarose gel normally do not pose a problem
2. Digest a 10 µL sample of the PCR product using the appropriate restriction enzyme in the specified restriction buffer in a final volume of 20 µL (*see* **Note 3**).
3. Carry out restriction digests for 4 h.
4. Resolve digested PCR products by agarose gel electrophoresis. PCR reactions for point mutations resulting in a restriction site loss or gain have been generally designed to produce restriction fragments that can be resolved on 2% agarose gels (**Fig. 2**). Mismatch primer mutation analysis normally generates PCR products that require resolution of the initial product and the product minus the PCR primer (20–44 bp). These shorter fragments need to be run on a 4% Nusieve or 15–20% acrylamide gel (**Fig. 3**).

3.5. Quantitative Determination of Point Mutation Levels

For most diagnostic purposes, mutation screening as outlined in **Subheadings 3.3.** and **3.4.** is sufficient (*see* **Note 5**). In cases where an accurate estimation of mutant levels is required such as in cybrid experiments using rho- lines or when tissue is analyzed that is likely to have very low levels of a particular mutation (for example, the 3243 mutation in blood from patients with diabetes and deafness *[16]*) a more quantitative and sensitive method is required. For this a radioactive PCR product is produced by last cycle PCR labeling (*see* **Note 4**).

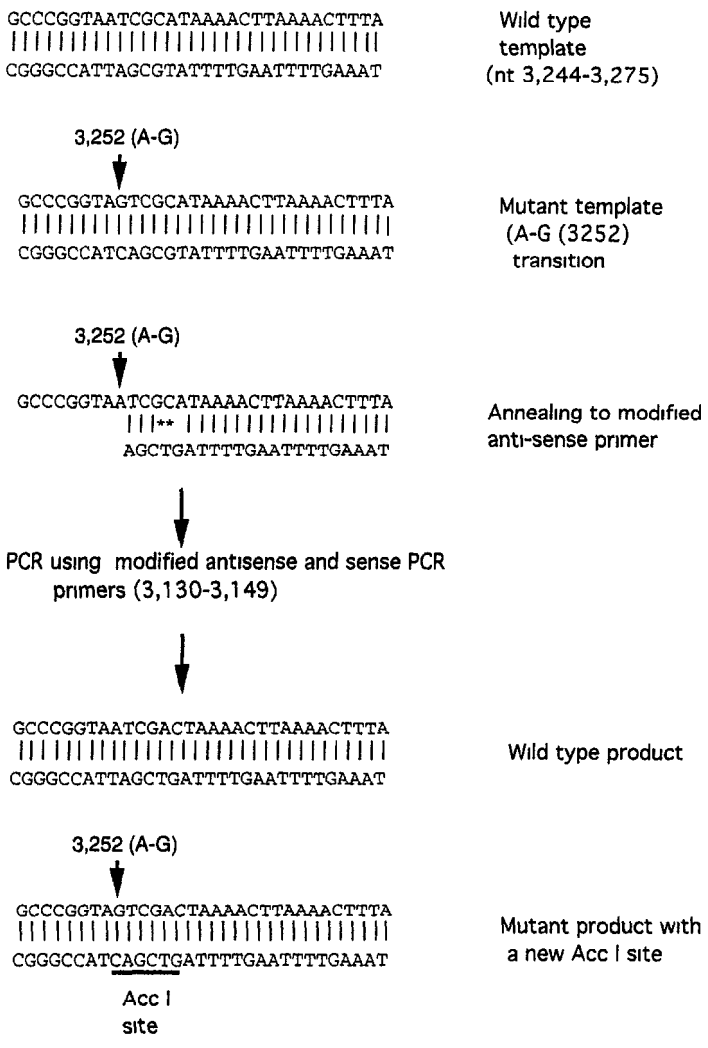

Fig 1. The principles of mismatch PCR analysis using the detection of the bp 3252 (A–G) transition in tRNA leucine (UUR) as an example

1. The region of interest is amplified by PCR, and 5 µL of the reaction is removed for analysis.
2. Add 5 µL of the following mix to each reaction to the remaining 45 µL of PCR reaction: 3 µL ^{32}P α dCTP (or any ^{32}P α dNTP), 8 µL 10X PCR buffer, 3 µL *Taq* polymerase (5U/µL), and 66 µL H_2O (enough for 16 PCR labeling reactions).
3. Carry out one cycle of PCR, extending for at least 2 min at 72°C
4. Digest 2 µL of each reaction with the appropriate restriction enzyme and run on a 5–8% polyacrylamide gel. Dry and autoradiograph.

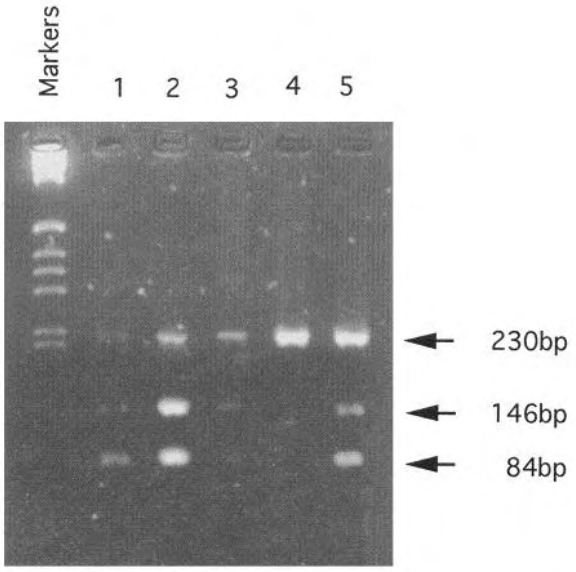

Fig. 2. *Mae* III digest of PCR products from LHON patients with the 11,778 mutation (G–A). Lanes (1–3,5) LHON patients with the 11,778 mutation and lane 4 normal control. The 146 bp and 84 bp bands reflect the level of mutant and the undigested 230 bp product wild–type mtDNA.

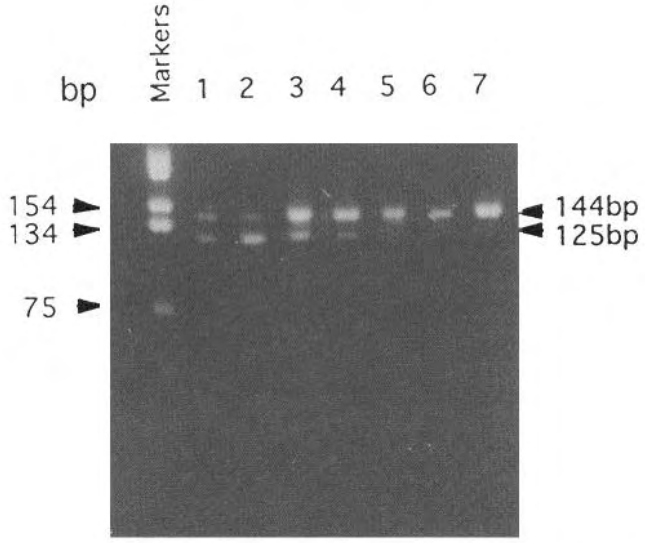

Fig. 3. 4% Nusieve gel of *Acc* I restriction digested mtDNA PCR products amplified using a mismatch primer for the 3252 (A–G) mutation. Lane 1 probands mother's muscle; lanes 2–4 probands muscle, blood, and lymphocytes; lanes 5–7 unaffected sisters muscle blood and fibroblasts. The 125-bp product reflects the level of mutant mtDNA and the undigested 144-bp product the wild–type mtDNA.

5. Mutant levels can be estimated using a phosphorimager or by cutting bands from the gel and counting with a scintillation counter

3.6. Screening for Novel Point Mutations

The majority of patients referred for mitochondrial mutation screening do not have any of the previously described mtDNA mutations and so are potential candidates for as yet undescribed mtDNA mutations (*see* **Note 6**). A number of PCR-based methods have been successfully used to screen for pathogenic mtDNA mutations *(27,18)*. However, before using such methods, a number of points are worth considering: Do patients have a clinical presentation and histochemistry/biochemistry suggestive of a mtDNA disorder? Is there any evidence of a maternal inheritance pattern? Does the severity of the phenotype vary between individuals in a pedigree? A positive answer to these questions may indicate that the mitochondrial disorder could be the result of a mtDNA mutation. However, even a strongly suggestive family history, biochemistry, and clinical phenotype will not guarantee the identification of a pathogenic mtDNA mutation. Even in laboratories where large numbers of patients are screened few new pathogenic mutations have been identified.

3.6.1. Direct PCR Sequencing

One of the most successful screening methods used for the identification of novel mtDNA mutations has been direct sequencing of PCR products. With current technologies such as magnetic beads and improvements in asymmetric PCR sequencing this technique is easily accessible. Most pathogenic mutations described so far are found in the 22 mtDNA tRNA genes that occupy a total of 1537 bp of the 16,569 bp mtDNA genome. Pathogenic mutations also tend to cluster in particular mtDNA tRNA genes e.g., tRNA leucine (UUR) *(28)*.

3.6.2. Screening Methods

A primary screening method which could deal with a number of patients simultaneously over large regions of the mitochondrial genome would offer significant advances over direct sequencing. Because of heteroplasmy the screening method must be able to reliably detect varying levels of mutant and also be able to distinguish between heteroplasmy and neutral polymorphisms. Two screening methods, chemical cleavage by mismatch (CCM) *(18)* and single-stranded conformational polymorphisms (SSCP) *(27)* have been used to screen mtDNA for mutations. Both methods offer significant advantages over direct sequencing. Using SSCP heteroduplex analysis Thomas et al. *(27)* were able to detect heteroplasmic mutations in a 409-bp fragment at positions 3251, 3260 and 3243. The method does not require the use of radioisotopes and detects only heteroplasmic disease-associated mutations and not

homoplasmic neutral polymorphisms. There is, however, a limit to the size of PCR fragments that can be screened by SSCP (300–500 bp). In addition to this, reaction conditions need to be optimized for each different PCR product screened and the method only appears to work reliably on a PhastSystem electrophoresis apparatus (Pharmacia). The CCM method described by Morten et al. *(18)* detected both known and new previously unidentified point mutations in a 293bp fragment down to levels of 5% (Morten, unpublished). The method was also able to screen PCR fragments up to 2 kb and reliably identified the positions of mutations for subsequent sequencing (Morten unpublished). Such an approach would allow the entire mitochondrial genome to be screened in 8–16 pieces. However, the CCM method does involve the use of toxic chemicals and radioisotopes and is unable to distinguish between heteroplasmy and homoplasmy. With the ever-increasing power of PCR now being able to amplify the whole mitochondrial genomes of heteroplasmic individuals with wild-type and deleted mtDNA (**Fig. 4**) (Marchington, unpublished), it is likely that it will not be long before a heteroplasmic screening method utilising one of the above mutation screens is capable of screening the entire mtDNA molecule for mutations in one reaction.

3.7. PCR Methods Used to Aid in the Diagnosis of mtDNA Mutations Detected by Southern Blot Analysis

Rearrangements of the mitochondrial genome such as deletions, duplications, and variable deletions are detected by southern blot analysis *(1,29,30)*. Deletions and duplications can be easily distinguished from the wild-type mtDNA molecule, but variable deletions can be problematic. Variable deletions show an autosomal dominant mode of transmission and are detected as multiple bands on a southern blot when linearized and probed with a mtDNA probe *(30,31)*. Levels of individual deletions are normally low and it is sometimes difficult to distinguish between bands that are artifacts resulting from the restriction digest and true variable deletions. A PCR approach can aid in the diagnosis of variable deletions in two ways: amplifying across the common deletion junction and long-range PCR amplifying the entire mtDNA molecule.

3.7.1. Detection of Variable Deletions by PCR Amplification Across the Common Deletion Junction

Methods should only be used to clarify southern blotting data when the autoradiographs suggest variable deletions may be present.

1. Muscle DNA is extracted as described in **Subheading 3.1.**
2. Set up PCR reactions (50 µL reaction) as follows: 5 µL 10X PCR buffer, 4 µL dNTPs (5 m*M*), 2.5 µL 5' CCAACACCTCTTTACAGTGA 3' Forward (8345–8364), 2.5 µL 5' GGTTTAGTATTGATTGTTAGCG 3'Reverse (14,568–

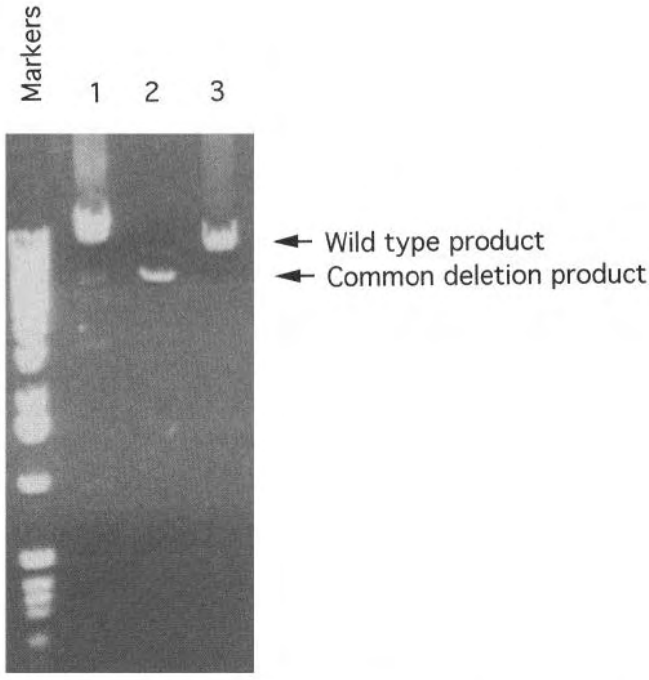

Fig. 4. Long-range PCR amplification of a patient with variable deletions (lane 1), a patient with high levels of the mtDNA common deletion (lane 2), and a wild-type mtDNA control (lane 3).

14,589), 0.5 µL *Taq* polymerase (5 U/µL), 1 µL DNA (50 ng/µL), and 34.5 µL H_20. use one of the components to "Hot start" the reaction at 80°C.

3. Cycling conditions:

1 Cycle	28 Cycles	1 Cycle
94°C for 5 min	94°C for 1 min	94°C for 1 min
50°C for 1 min	50°C for 1 min	50°C for 1 min
72°C for 1 min	72°C for 1 min	72°C for 3 min

3.7.2. Long Range PCR Amplification of Deletions and Wild-Type mtDNA Molecules

Recent advances in PCR technology now make it possible to amplify DNA molecules up to 44 kb in length ***(32)*** (*see* Chapter 9). The entire mtDNA genome has been amplified by a number of groups ***(33,34)*** and a number of amplification kits are available. In my laboratory, long-range PCR of mtDNA is still being evaluated and is not currently used for routine diagnosis. When developed further, long-range PCR amplification of the entire mtDNA molecule will offer the advantage of having the internal wild-type mtDNA product

as an internal standard allowing a direct assessment of the levels of deletions, duplications, and variable deletions.

4. Notes

1. The addition of salt prior to phenol extraction can result in a better separation of the aqueous and solvent phases It is, however, possible that this may result in a reduction in yield
2. Unless indicated, PCR reactions are carried out under standard conditions. If little template DNA is available then increasing the total number of cycles to 40 is normally sufficient to produce PCR products on most preparations A water control should be included in each PCR reaction to indicate PCR component contamination It is advisable to also include a positive control for the mutation that one is screening for, in order to determine restriction enzyme digestion efficiency.
3. Restriction digest conditions are not optimal for any of the enzymes used, but do provide adequate digestion for most mutation levels. If very low levels of mutant are expected, such as levels of the 3243 mutation in patients with diabetes and deafness, it is advisable to clean up the PCR product using a microspin column before digesting in the recommended restriction buffer. Using mismatch PCR primers can result in nonspecific PCR products which may interfere with the interpretation of subsequent restriction digests. Running an equivalent amount of uncut PCR product alongside each digest is normally sufficient to allow discrimination between true digestion products and uncut nonspecific PCR products.
4. Using a last cycle labeling strategy mutant levels of <1% can be detected This also prevents the underestimation of low levels of mutant (<20%) Low levels of mutant may be missed owing to heteroduplex formation (mutant /wild-type hybrids) during the later stages of the PCR reaction when conditions are less efficient for PCR. If a mutation is only present at low levels (<20%) and the PCR reaction is occurring suboptimally after the last cycle, single-stranded PCR products will be present that have not been amplified. These can either remain in the single stranded form or reanneal with a complementary strand. Because the predominate species in the reaction is the wild-type mtDNA, it is likely that all of the reannealling molecules will be either wild-type/wild-type or wild-type/mutant and, therefore, not be accessible to digestion. Under such conditions, mutation levels will be either under-represented or not detected at all.
5. One potential problem of screening for pathogenic mtDNA mutations is that they are usually heteroplasmic with mutant levels varying between tissues. Mutant levels are normally highest in affected tissues but can be either low or undetectable in tissues such as blood. It is therefore important to obtain an appropriate tissue before undertaking a mutation screening program DNA extracted from a muscle biopsy showing either ragged red fibers or cytochrome oxidase deficiency (or both) would be the tissue of choice The CCM and SSCP methods are capable of detecting low levels of mutations (<10%). Direct sequencing requires a much higher level of mutation to be present (>40%) to allow it to be distinguished from gel artefacts. With all methods, once a mutation has been identified a PCR method

such as described in **Subheadings 3.2.** and **3.3** can be set up to allow screening of other tissues from the patient and other family members.

6. One problem in determining whether a novel mtDNA point mutation is truely pathogenic is the high frequency of neutral polymorphisms that are found in mtDNA. Mutations occur approx 10 times more frequently in mtDNA than in nuclear DNA probably caused by the poor proofreading activity of the mtDNA polymerase and lack of a pyrimidine dimer repair system *(35)*. In the noncoding D-loop region and coding genes, mutations that do not impair function by causing a deleterious change such as changing a conserved amino acid can exist as neutral homoplasmic polymophisms. For a mutation to be considered pathogenic a number of the following criteria must be met
   a. Pathogenic point mutations must be shown to segregate with the disease phenotype within an individual, between individuals in a family and be confined to patients and their families rather than being found as polymorphisms in the general population.
   b Mutations are usually heteroplasmic
   c Pathogenic mutations must lie in regions that are highly conserved both within species and across species.
   d. There must be a correlation between mutant levels and mitochondrial malfunction. Correlating mutation levels in single fibres with reduced respiratory function by histochemistry or using rho zero (mtDNA free) cell lines to generate mtDNA mutant cybrids deficient in respiration are two of the most common methods of linking mutant levels to respiratory malfunction
7. It is advisable to amplify decreasing dilutions of DNA from a patient with a known amount of mtDNA deletion as a positive control If a normal region of mtDNA is amplified simultaneously, an approximate calculation of the levels of variable deletions can be made. More quantitative PCR approaches would be better using artificial templates containing both deletion and wild-type primer sequences so that a direct comparison can be made between deletion and wild type mtDNA levels. As low levels of deletions occur as a normal consequence of aging it is of vital importance to compare possible variable deletions patients to age-matched controls

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17

PCR Analysis of CD44 Variants in Tumors

John Bolodeoku

1. Introduction

The CD44 molecule is a transmembrane glycoprotein, it is an acidic, sulfated protein that contains multiple phosphoserine residues and several intrachain disulfide bonds. It is essentially composed of a 37-kDa protein core highly glycosylated by *N*- and *O*-linked sugars to form an 85–90-kDa product and is sometimes additionally linked to chondroitin sulfate side chains to produce a 180–220 kDa form on sodium dodecyl sulfate (SDS) gels *(1,2)*.

The human CD44 gene is located on the short arm of chromosome 11 p13 *(3,4)*. The genetic sequence encodes the CD44 protein backbone (standard form), which consists of proximal extracellular domains (exons 1–5 and 16), a transmembrane domain (exon 17), and a cytoplasmic domain (exons 18–20) In addition to the standard CD44 form, there are isoforms that arise from alternative splicing of ten variant exons (exons 6v1–15v10) into a site between exons 5 and 16. This alternative splicing mechanism allows the generation of different protein relatives with regions of similarity as well as variable functional domains *(5–9)*.

It was found that expression of isoforms of CD44 (CD44v) that contained sequences encoded by the exons 11v6–12v7 were able to confer metastatic potential to nonmetastatic rat tumor cells *(7)*, indicating that this derangement of CD44 expression may play a role in tumor metastasis. Following recognition that CD44 variant exons may play a role in the spread of rat tumor cells, many groups have screened human tumors for the expression of variant CD44 exons and introns, searching for correlation between expression profiles and prognostic parameters *(10–24)*. In these studies, the investigators have used the reverse transcription polymerase chain reaction (RT-PCR)/blot/hybridization technique that enables visualisation of the presence of certain variant exons and introns.

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In this chapter, detection of these alternatively spliced forms of CD44 in both human cell lines and human tissues using RT-PCR, followed by Southern blot hybridization is described.

2. Materials

2.1. RNA Extraction (see Notes 1 and 2)

1. Samples (surgically resected specimens and cell lines) for RNA extraction
2. Liquid nitrogen.
3. Denaturing solution: 4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0, 0.5% sarcosyl, 0 1 *M* 2-mercaptoethanol
4. Phenol
5. Chloroform-isoamyl alcohol (49:1)
6. Ethanol
7. Glycogen
8. Phosphate-buffered saline (PBS) (Sigma, St. Louis, MO).
9. Oligo (dT) celluose (Micro-fast track kit, Invitrogen, San Diego, CA) (*see* **Note 3**)
10. Cell scrapper.

2.2. RT-PCR

1. Superscript™ preamplification system (Gibco-BRL, Gaithersburg, MO)
2. cDNA cycle kit (Invitrogen).
3. Diethyl pyrocarbonate (DEPC) water
4. PCR primers (**Table 1**).
5. *Taq* DNA polymerase
6. Mineral oil.

2.3. Agarose Gel Electrophoresis and Southern Blotting

1. Agarose (electrophoresis grade).
2. 10X Tris-borate-ethylenediamine tetra-acetic acid (EDTA) (TBE) 0.9 *M* Tris base, 0.9 *M* boric acid, 20 m*M* EDTA, adjusted pH to 8.1–8.2 using solid boric acid
3. Ethidium bromide, 10 mg/mL in sterile distilled water.
4. 6X loading buffer· 0.25% bromophenol blue, 40% (w/v) sucrose in 1X TBE.
5. Whatman 3MM paper
6. Hybond N+ nylon membrane (Amersham International, Amersham, UK).
7. 0.4 *M* NaOH solution.
8. 2X SSC: Make up a 20X SSC stock solution and dilute to 2X SSC when required To make up a 20X SSC stock solution, dissolve 175.2 g of sodium chloride and 88.2 g of sodium citrate in a liter of distilled water

2.4. Hybridization and Detection

1. Prehybridization solution. 14.61 g of NaCl in 500 mL of gold hybridization buffer (Amersham) plus 25 g of blocking reagent (Amersham)
2. Hybridization oven.
3. ECL detection system (Amersham).

Table 1
List of Primers Used for RT-PCR Assays

Exon	Sequence of primers
Exon 3	**P1**:5'GACACATATTGCTTCAATGCTTCAGC-3'
Exon 18	**P4**:5'GATGCCAAGATGATCAGCCATTCTGGAAT-3'
Exon 7 (v2)	**E1**:5'TTGATGAGCACTAGTGCTACAGCA-3'
	E2:5'-CATTTGTGTTGTTGTGTGAA GATG-3'
Exon 8 (v3)	**V3**:5TACGTCTTCAAATACCATCTCAGC-3'
	AD1:5'-ACTGAGGTGTCTGTCTCTTT CATC-3'
Intron 9	**I4**:5'-GTAATGGGTTCTGCATATTT AATGAA-3'
	AI4:5'- CTGTGATGATGGTTAAATAC ACTG-3'
Exon 9b (v4)	**EX8**:5'TCAACCACACCACGGGCTTTTGAC-3'
	AEX8:5'AGTCATCCTTGTGGTTGTCTGAAG-3'
Exon 11 (v6)	**EX10**: 5'-TCCAGGCAACTCCTA-3'
	AEX10· 5'- CAGCTGTCCCTGTTG-3'
Exon 12 (v7)	**E3**:5'AGCCCAGAGGACAGTTCCTGG-3'
	E5:5'TCCTGCTTGATGACCTCGTCCCAT-3'
Exon 15 (v10)	**SD5**:5'GATGTCACAGGTGGAAGAAGAGAC-3'
	D5:5'TTCCTTCGTGTGTGGGTAATGAGA-3'

2.5. PCR

1 PCR primers Primer sequences are designed using information derived from the sequence of human CD44 (**Table 1**). The optimal primer length and thermal cycling was designed by Matsumara and Tarin *(10)*.
2. PCR reagents are provided in the Gibco BRL and Invitrogen kits.
3. Thermal cycler.

3. Methods

3.1. Sample Preparation and RNA Extraction

3.1.1. Human Tissue

1. Snap freeze fresh tissue samples (e.g., following surgical resection) in liquid nitrogen within 10–20 min of arrival. Keep in liquid nitrogen until use.
2. Homogenize tissue in the presence of 1 mL of denaturing solution
3. Add sequentially 0.1 mL of 2 *M* sodium acetate, pH 4.0, 1 mL phenol, and 0.2 mL of chloroform-isoamyl alcohol mixture (49:1). Mix thoroughly after the addition of each reagent
4. Shake the final suspension vigorously for 10 s and cool on ice for 15 min.
5. Centrifuge at 10,000*g* for 20 min at 4°C.
6. Transfer the aqueous phase to a fresh tube and mix with 2 volumes of ethanol and place at –20°C for at least 1 h to precipitate the RNA.
7 Centrifuge at 13,000*g* for 30 min at 4°C.

8. Dissolve RNA pellet in sterile nuclease free water and store at –70°C until required.

3.1.2. Human Cell Lines

1. Collect human cell lines grown in their respective culture media by scrapping with a cell scrapper.
2. Wash with PBS
3. Centrifuge the harvested cells into a pellet.
4. Extract total cellular RNA as described in **Subheading 3.1.1.**

3.1.3. Exfoliated Cells and Breast Fine Needle Aspirates

1. After smears have been made for routine cytological tests, resuspend cell remnants within the barrel of the syringe in cell culture medium.
2. Centrifuge at 2000–3000*g* for 5–15 min. Decant the supernatant and store the resulting pellet at –70°C.

3.2. Reverse Transcription

First-strand cDNA synthesis (RT), is performed using the following commercial kits SUPERSCRIPT Preamplification system for first-strand cDNA (Gibco BRL) and cDNA cycle kit (Invitrogen).

The protocol for using the Gibco BRL kit is as follows:

1. Mix all components and centrifuge briefly before use.
2. Add 5 μg of total RNA to an autoclaved 0.5-mL microcentrifuge tube.
3. Make up to 13μL with DEPC-treated water.
4. Add 1 μL of oligo(dT) to the tube and mix gently.
5. Heat the mixture at 70°C for 10 min and incubate on ice for 1 min.
6. Collect the contents in the tube by a brief centrifugation and add the following components:

Component	Volume, μL
10X Synthesis buffer	2
10 m*M* deoxyribonucleoside triphosphate (dNTP) mix	1
0.1 *M* dithiothreitol (DTT)	2
SuperScript II RT (200 U/μL)	1

7. Mix the reagents gently and collect by brief centrifugation.
8. Incubate for 10 min at room temperature
9. Incubate the mixture at 42°C for 50 min.
10. Terminate the reaction by placing on ice.
11. Collect the reaction mixture by brief centrifugation.
12. Add 1 μL of RNase H to the tube and incubate for 20 min at 37°C before proceeding to PCR.

The protocol for using the Invitrogen cDNA cycle kit is as follows:

1. Add 5 μg of total RNA to a sterile microcentrifuge tube.
2. Make up with sterile water to bring the total volume to 11.5 μL

3. Add 1 µL of oligo(dT) to this mixture
4. Heat the tube at 65°C for 10 min.
5. Centrifuge the tube briefly to collect the contents and add the following reagents in the order listed.

Component	Volume, µL
RNase inhibitor	1
5X RT buffer	4
100 m*M* dNTPs	1
80 m*M* sodium pyrophosphate	1
Avian myeloblastosis virus (AMV)	0.5

3.3. Polymerase Chain Reaction (see Notes 4 and 5)

The compositions of the following PCR reaction mixtures are in accordance to the suggestions of Gibco BRL and Invitrogen.

The GIBCO kit reactions are made up as follows:

Component	Volume (µL)
cDNA	2
10X Synthesis buffer	8
DEPC-treated water	68
Amplification primer 1	1
Amplification primer 2	1
5U/µL*Taq* DNA polymerase	1

The Invitrogen kit reactions are made up as follows:

Component	Volume, µL
cDNA	5
10X PCR buffer	5
100 m*M* dNTP	1
Amplification primer 1	1
Amplification primer 2	1
Bring volume to 50 µL with sterile water	36
Taq DNA polymerase (5U/µL)	1

1. Perform 30 cycles of PCR The cycle parameters are as follows: 94°C for one min, 55°C for one min, and 72°C for two min, with an initial hot-start procedure adopted.
2. Include negative controls in which there is no template cDNA in the reaction mixture with each batch.

3.4. Agarose Gel Electrophoresis

1. Prepare 0.5X TBE working solution by diluting the 10X TBE stock solution.
2. Fill the electrophoresis tank with the 0.5X TBE solution.
3 Prepare the gel by adding sufficient 0.25 g agarose to 50 mL of 0.5X TBE (*see* **Note 6**).
4. Heat in a microwave oven until the agarose is dissolved.
5. Allow the solution to cool to approx 50°C.

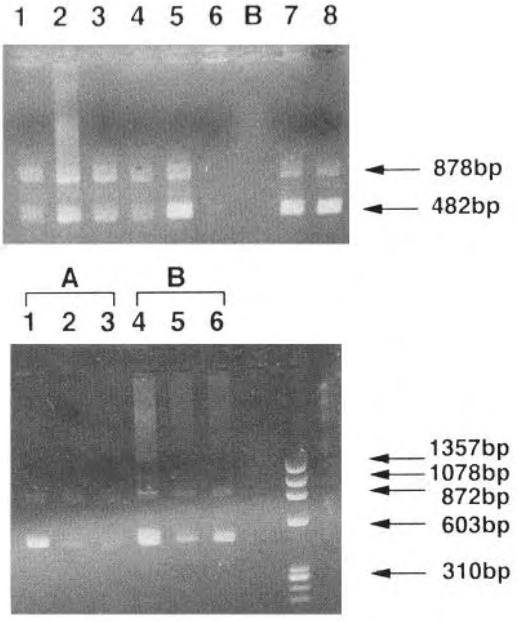

Fig. 1. (Top) RT-PCR analysis of CD44 gene transcription in fine needle aspirates in ethidium bromide stained gel (lanes 1–8). B-blank well. (Bottom) RT-PCR analysis of CD44 gene transcription in fine needle aspirates (lanes 1–3) and solid breast tissue (lanes 4–6).

6. While the gel is cooling, seal the edges of a clean UV transparent gel tray with autoclave tape.
7. Add ethidium bromide to the agarose solution to a concentration of 0.5 μg/mL and mix thoroughly.
8. Pour the warm agarose solution gently and evenly into the gel tray and insert a comb vertically into the solution.
9. Leave the gel to set for 30 min, and then carefully remove the autoclave tape.
10. Place the tray containing the gel into an electrophoresis tank filled with 0.5X TBE.
11. Prepare the DNA samples and molecular weight markers by mixing them with loading dye (5:1 [v/v]).
12. Load 10–15 μL of samples and markers into the wells (*see* **Note 7**).
13. Run the gel at 100 V until the dye front reaches the bottom of the gel.
14. Following electrophoresis, examine the gel for PCR products on a UV transilluminator and photograph with a Polaroid camera. Representative data are shown in **Fig. 1.**

3.5. Southern Blotting

1. Pour 500–1000 mL of a 0.4 *M* NaOH solution into a tank.
2. Place a glass plate across the top of the tank.
3. Cut three sheets Whatman 3MM filter paper to cover the glass plate and dip the paper in the form of a wick into the NaOH solution.

4 Put the agarose gel onto the filter paper, making sure that no air bubbles are trapped between the gel and the filter paper. Trim the unused areas of the gel away with a razor blade Cut the bottom left-hand corner of the gel to orient the gel during the succeeding operations. Cover the transfer apparatus with plastic wraps.
5. Cut a piece of Hybond N+ nylon membrane to exactly the same size of the gel. Place the membrane on the gel making sure there are no bubbles trapped between the gel and the nylon membrane.
6. Lay three sheets of Whatman 3MM filter paper on top of the nylon membrane, followed by a stack of absorbent paper.
7. Place a piece of glass and a weight of approx 500 g on the top of the absorbing paper.
8. Leave the gel to blot for more than 4 hours.
9. After blotting, mark the positions of the gel and the wells with a soft pencil and strip the membrane off the gel.
10. Wash the membrane for 5 min in 2X SSC and dry with filter paper.
11. Seal the membrane in a bag and store at 4°C until use.

3.6. Probe Generation by PCR

Prior to blot hybridization, the variant exon DNA probes should be generated.

1. Generate CD44 variant exon probes from a CD44 clone (*see* **Note 8**) by PCR (35 cycles, 94°C for 1 min, 55°C for 1 min, 72°C for 2 min) using the primers listed in Table 1 The positions to which the primers anneal are shown in **Fig. 2.** The reaction mixtures are the same as described in **Subheading 3.3.**

3.7. Hybridization of Southern Blot Filters

3.7.1. Prehybridization - Hybond N Filters

1. Place the filter in a hybridization bag.
2. Add prehybridization solution to the bag (10 mL of solution/100 cm^2 of filter).
3. Squeeze all the air bubbles out and seal the bag with a heat sealer.
4. Carry out prehybridization of the filter at 42°C for 20 min between two pieces of glass.

3.7.2. Labeling of Probes

1. While the membrane is undergoing prehybridization, label the DNA probe with peroxidase using the ECL direct nucleic acid labeling kit (Amersham).
2. Dilute the probe using water to a concentration of 10 ng/μL in an Eppendorf tube
3. Denature probe by heating at 95°C for 5 min (the minimum amount of DNA used is 100 ng in 10 μL).
4. Cool immediately on ice for 5 min and spin briefly in a microcentrifuge to collect the contents at the bottom of the tube.
5 Add an equivalent amount in volume of DNA labeling reagent (10 μL) to the cooled DNA.
6. Mix gently but thoroughly
7. Add an equivalent amount in volume of glutaraldehyde solution (10 μL) to the mixture, mix thoroughly, spin to collect the contents at the bottom of the tube and incubate for 10 minutes at 37°C.

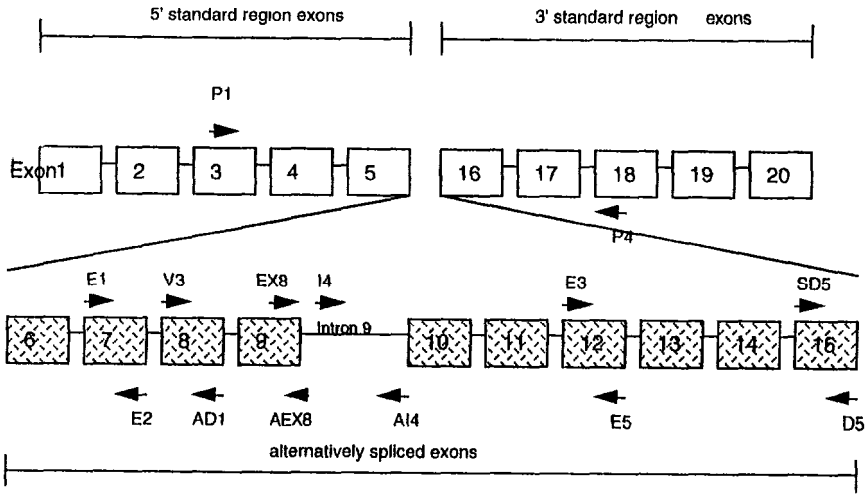

Fig. 2 Structure of the CD44 gene showing the positions to which the primers anneal. cDNA was amplified by PCR with P1 and P4 primers complementary to exon 3 and exon 17, respectively

3.7.3 Hybridization

1. After the prehybridization process, add the labeled probe to the prehybridization buffer (avoid direct contact with the membrane) and seal the plastic bag
2. Hybridize the membrane overnight in a rotisserie oven at 42°C.

3.7.4. Detection

1. Detect the chemiluminescent probes with the ECL detection system (Amersham) as recommended by the manufacturer's protocol Representative data are shown in Figs. 3–6.

4. Notes

1. RNA can be extracted using any established method but the acid phenol/guanidinium cell lysis method is used in the author's laboratory *(25)*.
2. As one is dealing with RNA, normal precautions such as the wearing of gloves, face masks, autoclaving instrument, and tubes are advised
3. mRNA isolation using (poly-T) - latex beads (Micro-fast track kit, Invitrogen) is used to extract RNA in circumstances where the expected sample size is limited. The principle of this method involves the extraction of polyadenylated RNA.
   a Lyse the cells in a detergent-based buffer containing protein degrader
   b. Incubate at 45°C and directly apply to oligo(dT) cellulose for absorption.
   c Wash nonpolyadenylated RNA, DNA, dissolved membranes, and cellular debris off the resin with high salt buffer, and tRNA and rRNA with a low salt buffer.
   d. Elute the polyadenylated RNA

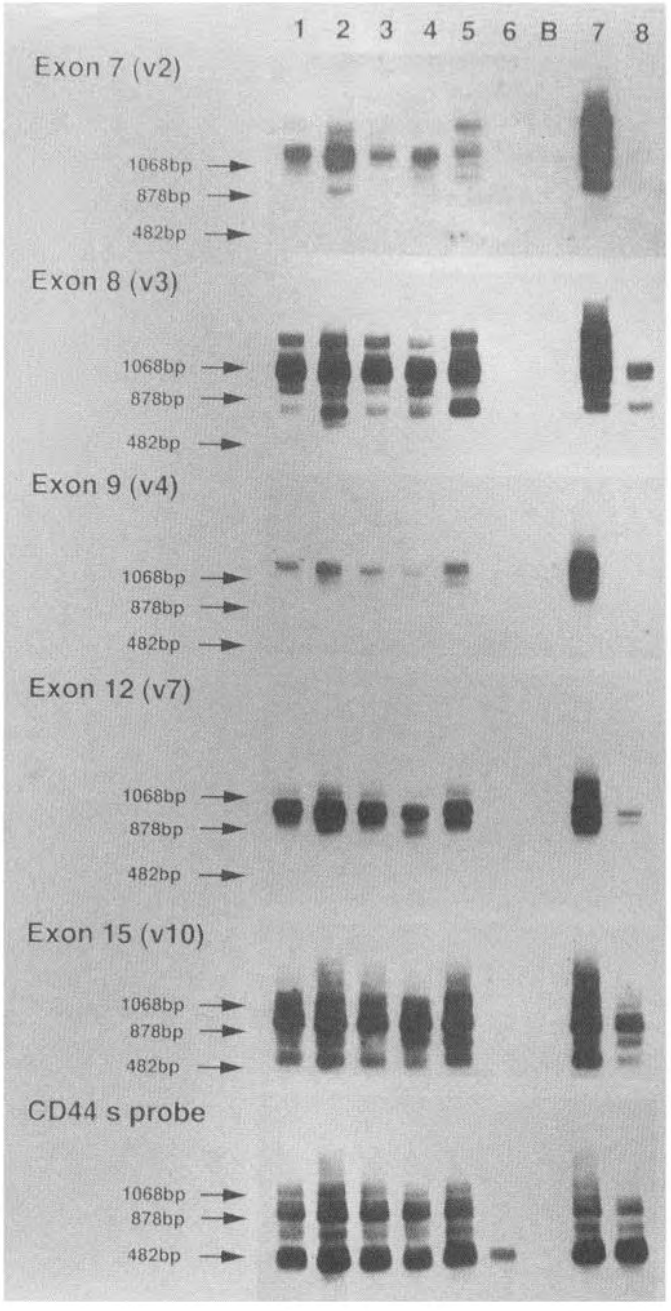

Fig. 3. RT-PCR/Southern blot hybridization analysis of CD44 gene transcription in breast fine needle aspirates.

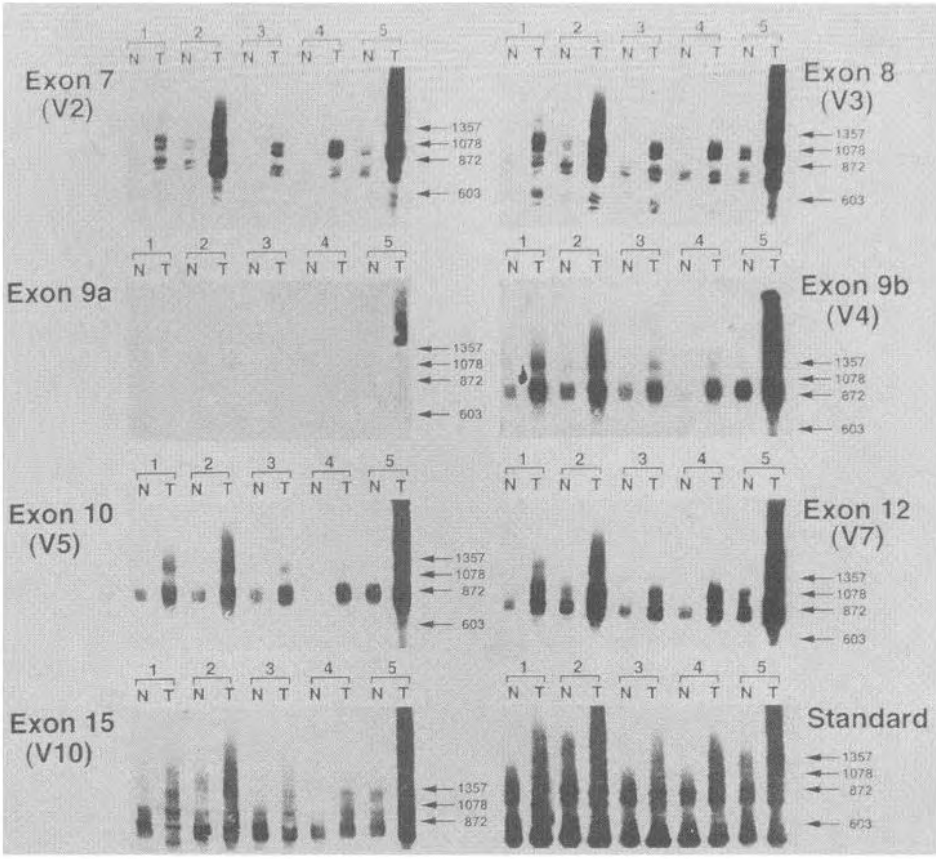

Fig. 4. RT-PCR/Southern blot hybridization analysis of CD44 gene transcription in solid breast tissue (N-normal and T-corresponding tumor).

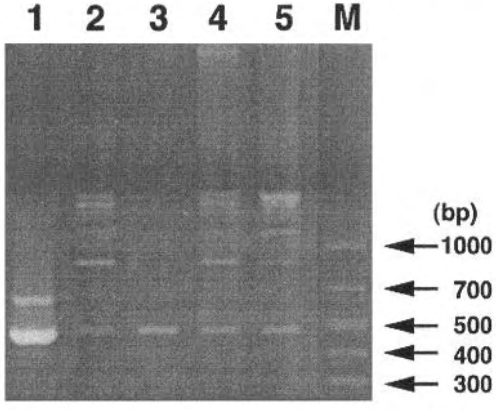

Fig. 5. RT-PCR analysis of CD44 gene transcription in different breast cancer cell lines (1-MDA-MB-435, 2-ZR-75-1, 3-Du4475, 4-MCF-7, 5-T47D).

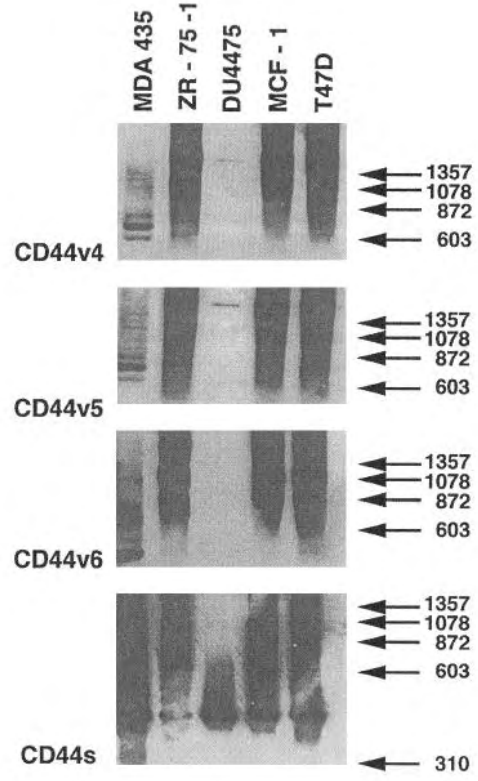

Fig. 6. RT-PCR/Southern blot hybridization analysis of CD44 gene transcription in different breast cancer cell lines (MDA-MB-435, ZR-75-1, Du4475, MCF-7, T47D).

The circumstances in which this method has been employed and found useful is in the examination of CD44 expression in exfoliated tumor cells from urine *(26,27)*, colonic washings *(28)* and breast fine needle aspirates biopsies *(29)*.

4. It is best to use between 5–10 μg of total RNA.
5. The PCR reaction can be made up to 50 or 100 μL and should be overlaid with mineral oil.
6. The amounts provided for making a 1.2% agarose gel are for casting a small gel, if making a large gel multiply all amounts by four.
7. The mineral oil should be aspirated completely before loading samples on gel.
8. The template for making the probes is generated by amplifying RNA from a breast cancer cell line ZR-75-1 with P1 and P4. The products run on a gel and the largest band is excised from the gel and ligated with a vector (Bluescript).

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18

Detection of Circulating Solid Tumor Cells by Reverse Transcriptase Polymerase Chain Reaction

Susan A. Burchill

1. Introduction

Despite advances in cancer treatment, disseminating disease continues to pose a major problem in clinical management. The dissemination of cancer cells around the body, or metastasis, involves multiple tumor–host interactions. The relationship between circulating tumor cells and development of secondary disease is not fully understood, though for cells to metastasize they must be capable of entering and surviving in peripheral blood or bone marrow. Although it is thought less than 0.01% of circulating tumor cells will successfully establish metastatic colonies *(1)*, a method to detect small numbers of such cells will allow evaluation of their role in the disease process. This may provide the clinician with a powerful tool to predict recurrence and relapse. In the longterm this may lead to improved clinical outcome by additional treatment of persistent disease following conventional therapy or early intervention in patients with micrometastatic disease and preclinical relapse.

A major advance in the detection of small numbers of circulating tumor cells occurred with the advent of the polymerase chain reaction (PCR) and the ability to amplify a specific region of DNA between defined oligonucleotide sequences *(2)*. This approach allows detection of one malignant cell in up to 10^7 normal cells, increasing the sensitivity of detection by an order of magnitude compared to immunocytochemistry *(3)*. PCR amplification of tumor-specific DNA sequences has been most successful in hematologic malignancies, where consistent and well-characterized molecular abnormalities have been described *(4–9)*. Such abnormalities are less common in solid tumors, and in these cases amplification of tissue-specific RNA has been used to detect circulating solid tumor cells *(10–17)*.

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Table 1
RNA Targets Used for RT–PCR Detection of Tumor Cells

Tumor type	Target for RT–PCR
Ewing's sarcoma, pPNET's	EWS–FLI1, EWS–ERG
Alveolar rhabdomyosarcoma	PAX3–FKHR, PAX7–FKHR
Prostate	Prostate–specific antigen, Prostate–membrane specific antigen
Breast/colorectal	Carcinoembryonic antigen
Neuroblastoma	Tyrosine hydroxylase, PGP 9.5
Melanoma	Tyrosinase, PGP 9.5, MAGE
Hepatoma	α–fetoprotein, Albumin
Epithelial	Cytokeratins

Reverse transcriptase polymerase chain reaction (RT-PCR) is increasingly used for the detection of tumor cells in peripheral blood, bone marrow, lymph nodes and peripheral stem cell harvests. Amplification of RNA sequences to detect circulating tumor cells can offer some advantages over DNA–PCR; even where chromosomal abnormalities have been identified RNA–PCR is often the method of choice. The position of chromosome translocation breakpoints can vary between tumors and may be scattered in many kilobases of DNA involving either chromosome. Splicing exons during transcription of the chimeric gene, generating a single RNA fusion product, can reduce the length of target nucleotides to be examined. Therefore, the translocation can often be detected in target cells using a single primer pair. A second advantage is its broader application, allowing identification of tumor cells with no known consistent chromosomal abnormalities by expression of tissue–specific RNA. This is a powerful approach, providing the sample to be analyzed for tumor cells does not express the tissue-specific target. Despite the rarity of absolute tissue-specific gene expression, several RNAs have been successfully used for the detection of common carcinomas in blood, bone marrow, peripheral blood stem cells (PBSCs), and lymph nodes (**Table 1**).

1.1. Sample Preparation and Isolation of RNA

RNA-PCR differs from standard PCR in that it uses RNA as the template rather than DNA (*see* Chapter 14). Transcribed mRNA is chemically less stable than DNA, rRNA or tRNA. Although this is beneficial for a messenger molecule, degradation of mRNA can pose a problem when analyzing biological samples that have not been rapidly or correctly processed. This is particularly difficult when analyzing circulating tumor cells in blood, bone marrow or PBSCs, since red blood cells contain high levels of RNAses that once released

Table 2
Standard Conditions for Conversion of RNA to cDNA by Reverse Transcriptase and Amplification of cDNA by Polymerase Chain Reaction

	RT	PCR
Reaction buffer	10 m*M* Tris HCl 50 m*M* KCl	10 m*M* Tris HCl 50 m*M* KCl
$MgCl_2$	8 m*M*	1 5 m*M*
dNTP's	1 m*M*	200 μ*M*
Enzyme	Mo–MLV	*Taq* DNA polymerase
Primer	Random hexamer	Specific primers
RNA guard	Yes	No

will rapidly degrade mRNA *(18)*. To minimize degradation, RNA should be extracted immediately or samples transferred to a –80°C freezer for storage.

The proportion of mRNA within a cell is only 1–5% of the total RNA, therefore, some sensitivity advantage can be gained by isolating mRNA (Burchill, unpublished observation). However, in most cases extraction of total RNA is adequate for RT-PCR. To minimize degradation of RNA, samples are lysed in guanidinium thiocyanate, a strong protein denaturant that at a concentration of 4 *M* and higher inactivates RNAses *(19)*. Solutions, glassware and plastics that will come in contact with RNA are treated with diethylpyrocarbonate (DEPC), which denatures RNAses. The quality and quantity of RNA isolated from a sample should be assessed before analysis by RT-PCR.

1.2. Reverse Transcription of RNA to cDNA

Since DNA polymerase cannot directly amplify RNA sequences, purified RNA must be converted to cDNA using reverse transcriptase and a selected primer (**Fig. 1**). The reverse transcriptase enzyme uses single stranded mRNA as a template to produce cDNA under the appropriate conditions. Random hexanucleotide primers, oligo dT (for eukaryotic cells) or the 3' antisense gene-specific primer employed in the PCR can be used to produce cDNA from the single stranded mRNA. To protect RNA from degradation by ribonucleases, RNAse inhibitors are included in the RT reaction (**Table 2**).

1.3. Amplification of cDNA

PCR allows amplification of a defined region of DNA, which may be up to several thousand base pairs in length (**Fig. 1**) *(2)*. Amplification of a denatured genomic or complementary DNA template is achieved using a thermostable DNA polymerase, which synthesizes the complementary strand by addition of

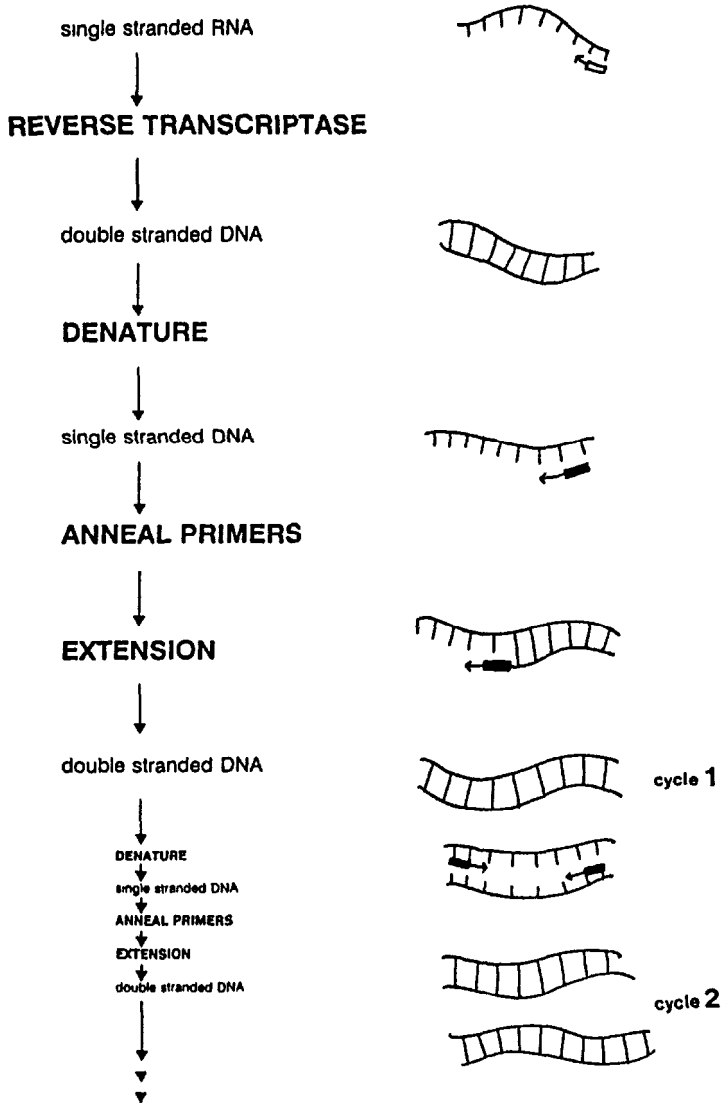

Fig. 1. Reverse transcriptase polymerase chain reaction. Single-stranded RNA is converted to double-stranded cDNA, using RT and a selected primer PCR, using thermostable DNA polymerase and sequence-specific primers, is employed to amplify a specific region of denatured DNA. DNA is synthesized from the 3' end following annealing of selected primers. With successive cycles of denaturation, annealing and extension, the amount of DNA increases. Amplification of target DNA sequences is specific, defined by the primer sequences and the annealing temperature

nucleotides at the 3' end of selected short oligonucleotide primers. The amplification of target DNA sequences is specific, defined by the selection of primer sequences and the annealing temperature. DNA sequence information is required for amplification of a target region, to allow identification of sequences to which oligonucleotide primers can be designed. The primers are selected to anneal to opposite strands of denatured DNA so that extension occurs in the 5' and 3' direction of the target DNA. The polymerase chain reaction usually consists of three phases, denaturation, annealing and extension (*see* Chapter 1 and summary below).

1.3.1. Denaturation

DNA usually exists as a double stranded helix, this must be denatured into two component strands for amplification. Heating DNA to 90–95°C breaks the hydrogen bonding between the two strands to give two single components.

1.3.2. Annealing

Once DNA is denatured the primers can recognize their complementary sequences and if the temperature is lowered sufficiently they will anneal to the single stranded DNA. The optimum annealing temperature may be between 50 and 75°C, depending on the A and T, G and C content of the primers.

1.3.3. Extension

Synthesis of the cDNA strand occurs by incorporation of free dNTPs by DNA polymerase at approximately 72°C. With one cycle of PCR a single double-stranded DNA molecule is amplified to produce two. These two new DNA strands now become the templates for the second PCR cycle, producing four double DNA strands, hence the chain reaction producing a doubling in DNA with each cycle (**Fig. 1**). The number of PCR cycles is repeated many times, resulting in an exponential increase in the amount of DNA synthesized.

1.4. Analysis of RT-PCR Products

Following amplification of target sequences, products of RT-PCR are analyzed by agarose gel electrophoresis and staining of doubled-stranded DNA RT-PCR product with ethidium bromide. Ethidium bromide chelates in the helix of double-stranded DNA and fluoresces under UV light. The use of Southern blotting or liquid hybridization can increase the sensitivity and specificity of detection. The identity of RT-PCR products should be confirmed by direct sequence analysis and comparison with the known target sequence when establishing the method for a new target.

1.5. Limitations and Future Applications

RT-PCR technology has made it possible to detect small numbers of tumor cells through amplification of specific nucleotide sequences in RNA, with a sensitivity that exceeds all other currently available methods. The method has been used for sensitive staging of patients and to detect residual disease. Whether staging of increased sensitivity will be of clinical significance is not clear, though most studies show a strong correlation with stage by conventional criteria and a proportion of patients with localized disease who have detectable tumor cells in blood or bone marrow. In tests for residual disease, mostly in hematological disorders, the detection of circulating tumor cells usually correlates with relapse. Potentially, the method may be valuable to monitor current disease status and identify preclinical relapse. This may give the opportunity to intensify treatment in some patients with better effect, or assess alternative therapies such as immunologic manipulation and the use of biological response modifiers. However, for most patients more effective treatment is required before the detection of low numbers of circulating tumor cells will improve outcome.

Despite these potential advantages, the superior sensitivity of this test can lead to false positives. This and the detection of leaky transcription remain a challenge. The inclusion of appropriate controls in all analyses is absolutely essential. Like all current methods used for the assessment of disease status, sampling variations can also lead to false negatives. Therefore, consistent results in sequential samples are vital to reliably assess tissues for tumor cells. Application of the method to detect disease throughout therapy should be interpreted with particular care, since neutropenia following treatment results in a decrease of total RNA and some treatments can reduce the level of transcription within single cells. These variations in transcription rates can make interpretation of results difficult in the absence of appropriate controls. Similarly, variation in transcription rates between different tumor cells will lead to different sensitivities of detection between individuals, and, if expression of the tissue–specific target is heterogenous between tumor cells, even within an individual. Finally, although the technique can be used to detect circulating tumor cells with great sensitivity and specificity, it will not measure cell number.

In summary, RT-PCR is increasingly used for the detection of subclinical disease. This may ultimately lead to a redefinition of what constitutes remission, but unfortunately the low efficacy of treatment for many malignancies means this would not immediately translate into improved outcome for patients. However, as new therapies evolve so molecular information may be useful in monitoring their impact. The method may also be useful to detect contaminating tumor cells in PBSCs and bone marrow used for transplantation. Studies to assess the clinical significance of detecting low numbers of circulating tumor cells will be particularly important in the next few years to evaluate the clinical significance of this technique.

2. Materials

As with all PCR studies the use of RT-PCR requires good laboratory procedures and practice. These include prealiquoting all solutions into small amounts, so a single aliquot is used in one RT-PCR and then discarded. This reduces stability problems associated with freeze/thawing solutions and the risk of contamination between experiments. Reaction components are mixed on ice and enzymes in particular removed from the freezer for the minimum amount of time. The use of designated pipets for each of the procedures also reduces the risks of contamination.

The sensitivity of this technique makes it vulnerable to small variations in volumes, consequently a master mix is prepared of all reagents to standardize the procedure as much as possible.

Water used is either distilled (dH_2O), double distilled (ddH_2O) or double distilled treated with DEPC (DEPC ddH_2O).

Sources of materials are given below, though these are examples and not exclusive. Unless stated otherwise, standard grade chemicals are suitable.

2.1. Sample Preparation and Isolation of RNA

Care should be taken to minimize exposure to biological hazards when handling blood, bone marrow, or PBSC samples. This would include wearing gloves and laboratory coats and following the guidelines for good laboratory practice.

1. Ultraspec™: RNA is extracted from biological materials using Ultraspec available from Biogenesis (Poole, UK). Ultraspec is a 14 *M* solution of guanidine salts and urea, containing phenol. It is stable for up to 12 months if stored in the dark at 4°C At 4°C the reagent can solidify, so the solution should be brought to room temperature and shaken before use Aliquoting Ultraspec into a small volume e.g., 8 mL for each 2-mL blood sample will avoid repeated freeze thawing and contamination risks
   Ultraspec contains poison (phenol) and irritant (guanidine salts). Phenol is a carcinogen and mutagen that can cause severe burns It can be fatal and should be handled wearing gloves and eye protection.
2. Diethyl pyrocarbonate (DEPC): DEPC will destroy RNAses and is used to treat all water and tubes used during RNA extraction and RT-PCR. It should be stored in the dark under dry conditions at 4°C. It is an irritant and toxic, demanding care when handling.
   DEPC treatment of water: DEPC is used at 0.1% e.g., to 1 L of double distilled water add 1 mL of DEPC, incubate at 37°C for 1 h. Tubes used during RNA extraction and RT-PCR are treated with DEPC by incubating in double distilled water containing 0.1% of DEPC for 1 h After treatment solutions and tubes are autoclaved to destroy residual DEPC, which if not inactivated will inhibit reverse transcriptase.
3. 3 *M* sodium acetate, pH 5.2. sodium acetate is used to precipitate RNA and oligonucleotides. It can be stored at room temperature, though should be protected

from moisture. To prepare, dissolve 40.81 g of sodium acetate $3H_2O$ in 80 mL of ddH_2O Adjust the pH to 5.2 with glacial acetic acid and make the final volume up to 100 mL with ddH_2O Dispense into 10 mL aliquots and autoclave

4. Loading buffer A to analyze RNA quality, 5X stock. 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol. Make up in dH_2O. Store at room temperature.
5 Ethanol
6. Chloroform.
7 Isopropanol.

Analar grade reagents should be used for 5, 6, and 7. A small bottle of each should be maintained specifically for RNA extractions to avoid contamination with RNAses. All are irritants. Chloroform is a carcinogen and should be handled with caution, wearing gloves and eye protection. Storage in a metal chemical bin is recommended.

2.2. Reverse Transcription of RNA to cDNA

1. 10X Reaction buffer: 100 m*M* Tris-HCl and 500 m*M* KCl, pH 8.3
   Available commercially from Perkin-Elmer (Norwalk, CT) as an autoclaved solution. Aliquot in 20 µL and store at –20°C. A single aliquot is used to give a final concentration of 10 m*M* Tris-HCl and 50 m*M* KCl in each experiment
2. Deoxynucleoside triphosphates (dNTPs). Ultrapure 2' deoxynucleoside 5'–triphosphates are available as 100 m*M* solution in water, pH 7.5 (Pharmacia Biotech, St Albans, UK) Prepare a 10 m*M* stock solution of all dNTPs by adding equal volumes of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) stock and diluting in DEPC-treated ddH_2O, e.g., 100 µL of each dNTP (combined volume = 400 µL) and add 600 µL of DEPC ddH_2O. Store at –20°C in small volumes (e.g., 50 µL).
3. Random primers: Commercially available from Gibco BRL (Gathersburg, MD) at a concentration of 0.09 OD U/µL in 3 m*M* Tris-HCl (pH 7.0), 0.2 m*M* EDTA Oligonucleotide sequences are largely hexamers. Store at –20°C
4. RNAguard: RNAse inhibitor derived from human placenta, commercially available from Pharmacia Biotech at a concentration of 37,000 U/mL. Store at –20°C.
5. 1 *M* Magnesium chloride: Molecular biology grade commercial preparation from Sigma (St. Louis, MO). Filter sterilized. RNAse, DNAse and protease free. Aliquot to 20 µL and store at –20°C.
6 Moloney murine leukemia virus reverse transcriptase: Available from Pharmacia Biotech at a concentration of 15,000–20,000 U/mL in 50 m*M* Tris-HCl (pH 8.3), 1 m*M* EDTA, 5 m*M* DTT, 0.1% Triton X–100, 100 m*M* NaCl and 50% glycerol Supplied in 1000 U aliquots. Stored at –20°C
7 Stock solution A for one RT reaction:

DEPC ddH_2O	2.57 µL
10X reaction buffer	2.00 µL
10 m*M* dNTP	2.00 µL

RNAguard	0.50 µL
100 m*M* $MgCl_2$	1.60 µL
Random primers	0.50 µL
RT enzyme	0.83 µL (10 U)
RT final concentrations	$MgCl_2$ = 8 m*M*, dNTP = 1 m*M*

2.3. Amplification of cDNA

1. Reaction buffer, $MgCl_2$ and dNTPs: source, stock and storage as for reverse transcriptase reaction.
2. Primers: Pair of single–stranded oligonucleotide primers (A and B) complementary to DNA flanking the region to be amplified. Primers are stored aqueous at –20°C; for longterm storage primers are stable precipitated in 1/10th vol sodium acetate and two volumes of ethanol.
   Primers are synthesized on an Applied Biosystems DNA/RNA synthesizer (Applied Biosystems Ltd, Warrington, UK); the primer yield and quality are tested by UV spectrophotometry and gel electrophoresis. Primers are available commercially e.g., from Perkin-Elmer.
3. DNA polymerase. Ampli*Taq* DNA polymerase from Perkin-Elmer is a thermostable DNA polymerase, encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene which has been inserted into an *Escherichia coli* host. The enzyme is available in vials containing 250 U at a concentration of 5 U/µL. The enzyme should be stored at –20°C and will remain stable under these conditions for several months. For each batch a control date is given, after which the enzyme may have lost activity
4. Wax pellets: Paraffin wax pellets from BDH (Dorset, UK) are used. These pellets are supplied in 1 kg quantities To limit the risk of contamination pellets are aliquoted (50–100 pellets/tube). The pellets are stored at room temperature.
5. Stock solution A for PCR

DEPC ddH_2O	15 µL (to make final volume of stock solution A 30 µL)
10X reaction buffer	3 0 µL
primer A	80 pmol (volume dependent on primer concentration)
primer B	80 pmol (volume dependent on primer concentration)

6. Stock solution B for PCR

DEPC ddH_2O	44.5 µL
10X reaction buffer	5.0 µL
Ampli*Taq*	0.5 µL
PCR final concentrations:	$MgCl_2$ = 1 6 m*M*, dNTP = 200 µ*M*

2.4. Analysis of RT-PCR Products

1. Agarose: Ultrapure electrophoresis grade agarose (Gibco BRL) is used for electrophoresis. Agarose powder is stored at room temperature under cool, dry conditions.

2. Loading buffer B for analysis of RT-PCR products (5X stock): 10% Ficoll, 0.05% bromophenol blue, 0.25% orange G, 0.5% sodium dodecyl sulphate (SDS). Make up in dH_2O. Store at room temperature.
3. Tris borate buffer (TBE): To prepare 1 L of 5X stock solution. 54 g Tris base, 27.5 g boric acid, 20 mL 0.5 *M* EDTA, pH 8.0
   Working strength for agarose gel electrophoresis is 0.5X or 1X, diluted in dH_2O
   Store solution at room temperature in clear bottles. If a white precipitate forms in any batches then discard and make new stock. 5X TBE stock solutions are usually stable at room temperature for several months
4. EDTA: To prepare 0.5 *M* EDTA stock (pH 8.0) add 18.61 g of disodium ethylenediaminetetra-acetate $2H_2O$ to 80 mL of dH_2O. Stir on a magnetic stirrer and adjust to pH 8.0 with sodium hydroxide pellets Aliquot and sterilize by autoclaving. Na_2EDTA will not go into solution unless the pH is approx 8 0
5. Ethidium bromide. 2,7 diamino-10-ethyl-9-phenyl-phenanthridinium bromide is available as an aqueous solution at a concentration of 10 mg/mL Store at room temperature or 4°C. Ethidium bromide is an irritant and frameshift mutagen that should be handled with care. Always wear gloves and eye protection when handling.
6. Molecular weight markers: A wide range of DNA molecular weight markers are commercially available and the choice is dependent on the expected size of the RT-PCR product A small range molecular weight marker in routine use is ΦX 174 RF DNA/*Hae* III (Gibco BRL) and an example of a broader range marker is the 123 bp ladder (Gibco BRL). Markers are usually provided at a concentration of 0.5 to 1 μg DNA/μL of storage buffer. Common storage buffers used are 10 m*M* Tris–HCl (pH 7.4), 5 m*M* NaCl, 0.1 m*M* EDTA or 10 m*M* Tris–HCl (pH 7.4), 1 m*M* EDTA (pH 8.0).
   For all irritants, carcinogens and mutagens, do not get on skin and avoid breathing vapor. In case of contact, immediately flush eyes and skin with copious amounts of saline and water for at least 15 min It may be necessary to seek medical attention.

3. Methods

Gloves and laboratory coats are worn at all times during the following procedures; to minimize exposure to biological and chemical hazards, and to reduce degradation or contamination of RNA from RNAses on hands and contamination from coats.

3.1. Sample Preparation and Isolation of RNA

1. Collect blood samples into polypropylene tubes (15 mL) containing EDTA (10 m*M*; pH 8.0) and mix. Ideally take three 2-mL samples at any one time (*see* **Note 1**).
2. To 2 mL of blood add 8 mL of Ultraspec, mix by inversion of the tube and transfer to –80°C freezer until RNA extraction (*see* **Note 2**)
3. Remove blood sample from freezer, take to room designated for extraction of RNA (*see* **Note 3**) and allow to defrost (*see* **Note 2**). Homogenize blood in Ultraspec by pipeting up and down using a sterile disposable plastic pipet.

4. Add 2 mL of chloroform to the homogenate and shake samples vigorously for 30 s.
5. Stand on ice for 15 min.
6. Spin samples at 10,000g, 4°C, for 15 min.
7. Take the aqueous phase (upper layer) and transfer to a new polypropylene tube, add an equal volume of isopropanol and stand on ice for 30 min (*see* **Note 4**)
8. Centrifuge samples for 15 min at 10,000g, 4°C. RNA forms a white pellet.
9. Remove supernatant and discard. Add 1 mL of 75% ethanol to the pellet, disperse by pipeting and transfer to a DEPC-treated Eppendorf tube. Centrifuge at 13,000*g* for 15 min at 4°C. Repeat this wash step (*see* **Note 5**).
10. Air-dry RNA pellet and dissolve in DEPC ddH_2O (100 µL)
11. Acetate/ethanol precipitate RNA by adding 1/10th vol of sodium acetate (3 *M*) and 2 vol of ethanol, e.g., for 100 µL add 10 µL of sodium acetate and 200 µL of ethanol. Place at –20°C overnight or on dry ice at –80°C for 1 h. Centrifuge at 13,000g for 10 min, remove supernatant and wash pellet in 75% ethanol as above.
12. Repeat acetate/ethanol precipitate and wash step described in **Steps 10** and **11** (*see* **Note 6**).
13. Measure the amount and purity of recovered RNA by spectrophotometry Measure optical density of RNA at 260 nm and 280 nm. An optical density of one corresponds to approximately 40 µg/mL of RNA. The ratio between the readings at 260 and 280 nm provides an estimate of recovered RNA purity. Pure preparations of RNA will have a ratio of between 1.8 and 2.0 (*see* **Note 7**).
14. The quality of RNA is determined by separation in a 0.8% agarose gel and staining with ethidium bromide (0.5 µg/mL). To prepare an agarose gel *see* **Subheading 3.4.** Take 1 µg of RNA in a volume of 10 µL DEPC ddH_2O, add 2 µL of loading buffer A, mix and load onto agarose gel (*see* **Note 8**). Run gel at 50V in 1X TBE The identification of 28 and 18 s rRNA confirms the quality of the RNA; contaminating DNA in the sample can be seen as a high molecular weight product (**Fig. 2**) (*see* **Note 9**).

3.2. Reverse Transcription of RNA to cDNA

The following procedure is performed in a designated PCR room. RNA and components for RT reaction are handled in a controlled environment e.g., laminar flowhood or PCR work station (Microflow, Hampshire, UK). Each worker has a designated Howey–laboratory coat for use in the PCR room, to which there is restricted access by personnel. To reduce the risk of cross contamination from pipets and aerosols use plugged pipet tips (*see* **Note 10** and **Fig. 3**).

1. Aliquot target RNA (1 µg) dissolved in 10 µL of DEPC ddH_2O in a 0.5 mL DEPC-treated Eppendorf Heat for 5 min at 95°C to linearize RNA Place onto ice.
2. To RNA add 10 µL of stock buffer A Mix by pipeting. Secure lid to prevent forming aerosols (*see* **Note 11**).
3. Incubate at 37°C for 1 h on heating block
4. Heat to 95°C for 5 min to inactivate reverse transcriptase enzyme (*see* **Note 12**).

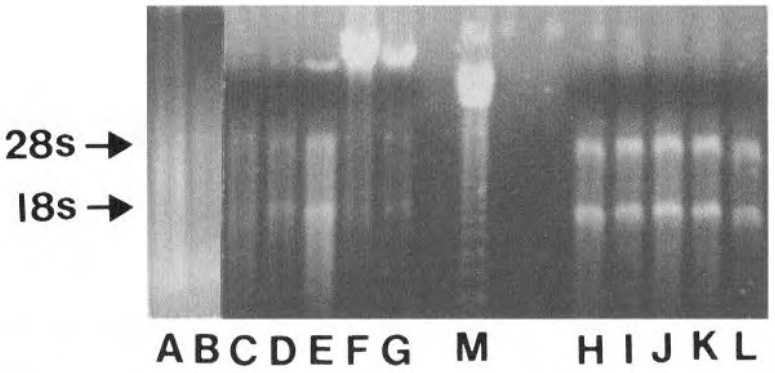

Fig. 2. Analysis of RNA by agarose gel electrophoresis. The quality and purity of isolated RNA is analyzed by electrophoretic separation in an agarose gel and staining with ethidium bromide. The figure shows separation of RNA (1 µg) extracted from 12 different patient samples. Bright 28s and 18s bands are readily identified in samples of intact RNA (lanes H, I, J, K, and L). Contaminating DNA is visible as a high molecular weight product (E, F, and G), the decreased intensity of 28s and 18s bands reflects the lower levels of RNA in these samples. Where RNA is degraded a bright ethidium bromide stained smear, toward the bottom of the gel, is seen (lanes A and B). M, molecular weight markers.

5. Pulse spin to remove condensation from tube lid.
6. Place on ice and go to amplification stage.
7. Alternatively, cDNA samples can be stored at –20°C before amplification.

3.3. Amplification of cDNA

In the PCR room:

1. Take 20 µL RT product and heat to 95°C for 5 min (*see* **Note 13**).
2. Cool quickly by placing Eppendorf on ice. It may be necessary to briefly spin to remove condensation from lid.
3. Mix 20 µL of RT solution with 30 µL of PCR buffer A (*see* **Note 14** and **Fig. 4**).
4. Place wax pellet on top of PCR solution and briefly heat at 90°C until wax pellet melts (approximately 30 s) (*see* **Note 15**).
5. Cool on ice until wax solidifies.
6. Overlay wax pellet with 50 µL of PCR solution B (*see* **Note 16**).
7. Place on PCR machine and heat to 95°C for 2 min to fully denature cDNA.
8. Amplify cDNA using the following PCR cycling parameters: 94°C for 30 s, 60°C for 60 s, 74°C for 60 s.
   Perform 30 cycles of PCR (*see* **Note 17** and **Fig. 5**).
9. Final extension of 74°C for 7 min.

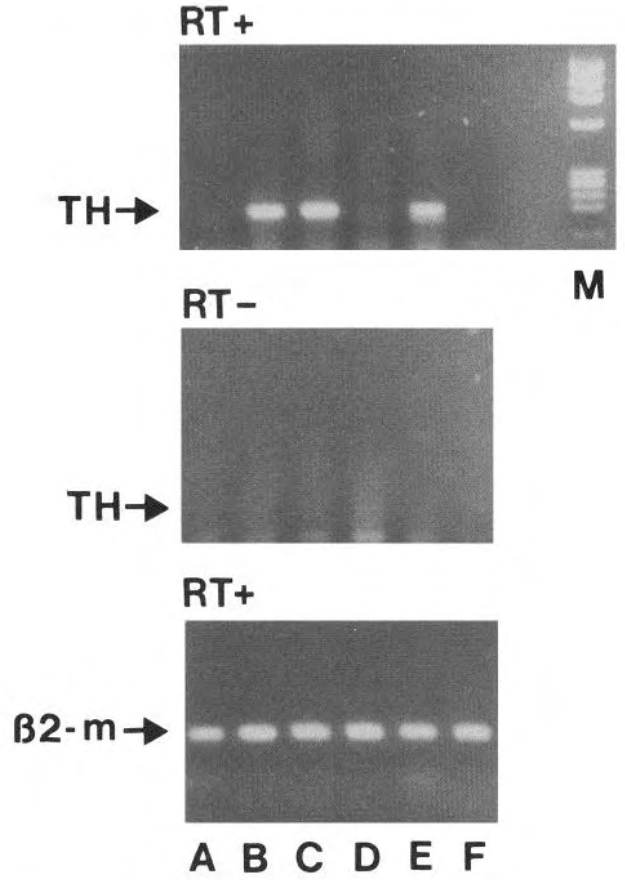

Fig. 3. RT-PCR analysis for tyrosine hydroxylase in six patient blood samples. RT-PCR for tyrosine hydroxylase mRNA has been used to identify circulating tumor cells in patients with neuroblastoma *(14)*. The panel labeled RT+ shows three samples positive for tyrosine hydroxylase mRNA (B, C, and E) and three negatives (A, E, and F). To control for specificity of amplification from RNA, for each sample an RT negative control (RT–ve) is included. No products are detected in the RT– samples. The quality of RNA for RT-PCR analysis is confirmed by RT-PCR for β2–microglobulin (β2 – m) (bottom RT+ panel). M, molecular weight markers

3.4. Analysis of RT-PCR Products

1. Separate RT-PCR products by electrophoresis in a 2% agarose gel in 1X TBE. For a 100 mL slab gel, mix 2.0 g of Ultrapure agarose with 1X TBE buffer in a conical perspex flask. Use a conical flask to allow adequate mixing of the agarose and 1X TBE. Once the agarose is well mixed, heat in a microwave on 80% heat setting for approximately 1 min, or until all the agarose has melted. Remove

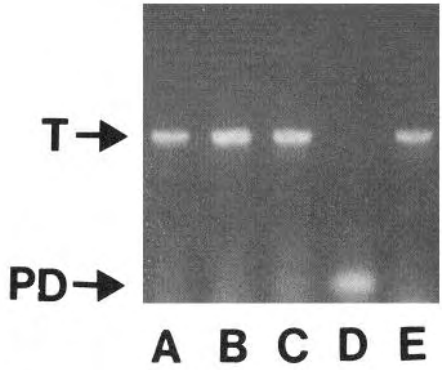

Fig. 4. Formation of primer dimers. RT-PCR for a target mRNA (T) in five patient blood samples. Samples A, B, C, and E are positive. No target mRNA was detected in sample D, the lack of target RNA resulting in formation of primer dimers (PD). Primer dimers will form when one primer anneals to the 3' end of the other, resulting in a decreased efficiency of amplification. Even when primer sequences are carefully selected to avoid complementary sequences at the 3' end, primer dimers may form if target RNA copy number is low or absent.

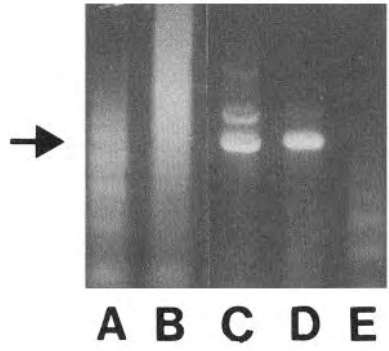

Fig. 5. Effect of annealing temperature and cDNA concentration on PCR efficiency. The amount of target RNA and consequently cDNA produced can affect the efficiency of amplification. Using an annealing temperature of 60°C, and amplification for 30 cycles RT-PCR for a target sequence (→) on 1 μg of RNA generates a single band (D). Increasing the amount of RNA to 5 μg (B) or 10 μg (A) no products were identified. The bright smear shown on the tracks reflects the high levels of cDNA produced during the RT reaction. The efficiency of the PCR is decreased with high cDNA concentrations. This effect can be reduced by RT-PCR analysis of poly A^+ RNA, rather than total RNA. Annealing temperature can also affect the efficiency of RT-PCR and should be optimised for each primer set. On 1 μg of total RNA, using an annealing temperature of 50°C, two dominant PCR products are detected (C). However, at 60°C a single PCR product of the expected size is produced (D). Increasing the annealing temperature to 70°C, no PCR product was detected (E).

the flask from the microwave and allow to cool to about 50°C. Once the agarose has cooled, add ethidium bromide (0.5 μg/mL) to the agarose, mix by swirling the flask and pour the agarose into the slab gel tray (the ends of which have been sealed with tape). Place comb into slots to produce wells (*see* **Note 18**). Avoid getting bubbles in the gel as these will interfere with electrophoresis. Allow approx 30 min for the gel to set (*see* **Note 19**).

Care must be taken as the agarose is very hot and producing steam when it is first removed from the microwave. It is advisable to wear safety goggles and heat resistance gloves when handling the flask. Do not leave the agarose in a microwave that is on unattended.

2. Remove an aliquot (30 μL) of RT-PCR product from the reaction tube into a clean Eppendorf. Use a fine gilson-tip to break through the solid wax layer, and use a second tip to remove the products. The use of two tips assures the sample obtained for analysis is free from contaminating wax; wax can cause samples to float from the well when loaded on the agarose gel. New, sterile tips must be used for each sample to avoid cross contamination of PCR products.
3. To the RT-PCR product (30 μL) add 6 μL of loading buffer B and mix by pipeting up and down into a tip using a gilson. Again use a new tip for each sample The RT-PCR product is now ready to be loaded on the gel. Run DNA molecular weight markers on the same gel to determine the size of RT-PCR products. Use 2 μL of ΦX 174 RF DNA/*Hae*III and 2 μL of 123 bp ladder added to 22 μL of dH_2O plus 4 μL of loading buffer B in each case.
4. Fill gel tank with 1X TBE buffer Remove tape from ends of gel tray and place into tank. Gel should be completely immersed in buffer. Slowly remove comb from gel, taking care to avoid damaging the wells. Damaged wells will result in uneven separation of DNA products in the gel or even worse loss of sample into the next well or between the gel and tray.
5. Electrophorese at 50–70 V for 2–3 h, until separation of orange G and bromophenol blue into two distinct bands.
6. Visualize gel under UV-light using a transilluminator. Molecular weight markers and RT-PCR products are seen as bright bands (**Fig. 3**) due to the fluorescence of the ethidium bromide intercalated between the double stranded DNA. Photograph gel on transilluminator using Polaroid ISO 3000/36° film (Amersham, Bucks, UK).

4. Notes

1. The analysis of tissues for tumor cells by RT-PCR, immunocytochemistry or conventional cytology is subject to sample variation, therefore it is advisable to analyze more than one sample at any time point. Collection of more than one sample will also safeguard against loss or deterioration.
2. EDTA or citrate are the anticoagulants of choice since these do not interfere with subsequent analysis, unlike heparin Collection of samples into polyethylene tubes allows direct transfer to –80°C and addition of guanidinium based solvents for RNA extraction. The addition of guanidinium salts to samples prior to freezing will further reduce degradation by increased inhibition of RNAses. If samples

are not frozen in guanidinium salts, they should be added to the frozen sample as soon as it is removed from the freezer to minimize degradation during defrosting

3. RNA extractions are performed in an isolated room to avoid contamination with genomic or cDNA's It is especially important RNA samples do not become contaminated with RT-PCR products for target of interest.
4. Absence of a clear supernatant layer would suggest the blood, bone marrow or PBSC sample was not adequately mixed and lysed in **step 3** in **Subheading 3.1.** Dividing the sample, adding an equal volume of fresh Ultraspec and repeating **steps 4, 5,** and **6** may improve cell lysis and protein degradation and allow isolation of RNA from valuable samples.
5 The amount of ethanol to be used for each wash step is dependent on the amount of RNA recovered. As a guide use 1 mL per 50 μg of RNA isolated
6 The amount of DEPC ddH_2O added is dependent on the amount of RNA recovered It is usual to aim for an RNA concentration of 1 mg/mL. RNA may be made up in DEPC ddH_2O containing an RNAse inhibitor such as RNAsin (Boehringer Mannheim, Lewes, UK) or RNAguard (Pharmacia Biotech) to protect against degradation. RNA in aqueous solution will degrade if not stored at –20°C RNA to be stored for longer than 3 mo should be stored under ethanol acetate precipitation. Add 1/10th vol of sodium acetate (3 *M*) and 2 vol of ethanol and store at –20°C.
7 Deviation from a ratio of 1 8 to 2 0 indicates contaminating protein or phenol, and will make an accurate quantitation of nucleic acid impossible. Optical density readings at 260 and 280 nm will not differentiate between DNA and RNA, though contaminating DNA can be seen when RNA is separated on an agarose gel (**Fig. 2**) Contaminating DNA can be reduced by treating isolated RNA with RNAse free DNAse (2 μg/mL for 10 min at 37°C)

   It is advisable to aliquot purified RNA for future use, thus avoiding freeze-thawing which will lead to degradation of RNA. RNA can be stored aqueous at –20°C in the short term, for storage longer than 3 mo add 1/10th volume of sodium acetate, two volumes of ethanol and store at –20°C.
8. A loading buffer has three functions; it will increase the density of the sample so it drops evenly into the well, the dye adds color to the sample simplifying the loading process and finally the dyes migrate to the anode in an electric field allowing visualization of separation. In agarose gels between 0 5 and 1.4% bromophenol blue migrates at approximately the same rate as linear double stranded 300 bp DNA, faster than xylene cyanol FF which migrates at a similar rate to linear double stranded 4 kb DNA.
9. RNA that is partially degraded may be useful for analysis by RT-PCR. Therefore, it is advisable to analyze an aliquot of RNA by RT-PCR for a ubiquitous species e.g., β2–microglobulin, β–actin or glyceraldehyde–3–phosphate dehydrogenase or a target mRNA known to be expressed in the target tissue (**Fig. 3**). This analysis is important to confirm if the results for the target sequence are true i e., if no signal is seen with the target sequence, amplification for a control gene will support absence of specific target gene expression However, the lack of

target gene expression in such a case may reflect a failure in amplification due to contaminating products such as guanidinium salts or phenol, or completely degraded RNA. An additional problem with RNA samples extracted from blood, bone marrow or PBSCs can be coprecipitation of porphyrin compounds derived from heme, which can inhibit DNA polymerase activity *(21)*.

10. RT-PCR is an extremely sensitive technique, allowing detection of a single molecule. The great sensitivity of the technique can also be its downfall, the opportunities for contamination being great. To minimize contamination, a designated room is used for the set up of RT-PCR. All manipulations are performed in a controlled clean–air environment e.g., laminar flowhood, PCR bubble or a PCR workstation Laminar flowhoods or PCR workstations with facilities for UV irradiation are particularly useful, allowing the opportunity to eradicate contaminating surface and airborne DNA between procedures.
    The inclusion of relevant controls is important to detect contamination or amplification from genomic DNA, and positive controls to ensure the reaction has occurred successfully and with reproducible sensitivity. To control for amplification from contaminating DNA, for each RNA sample use a reverse transcriptase negative control (**Fig. 3**, RT–ve). All components of the RT stock buffer A are included except for the reverse transcriptase enzyme (which is replaced with 0 5 μL of DEPC ddH_2O). Water negative controls, in which RNA is replaced with DEPC ddH_2O, are used to control for contamination of solutions. Positive controls should include RNA extracted from a cell line or tissue known to express the target sequence. It is usual to include several concentrations e.g., 1 μg, 1 ng, 1 pg to evaluate amplification efficiency. A second positive control should be "spiked" blood or bone marrow samples. Known numbers of cells expressing the target sequence are added to a fixed volume of blood or bone marrow from a healthy volunteer e.g., 10, 100, and 1000 cells into 1 mL of blood. RNA extracted from these spikes is included in each experiment to confirm sensitivity of the amplification. The use of an internal control for a ubiquitously expressed gene to evaluate the suitability of RNA for amplification has been discussed in **Note 9**, this control can also be used for normalization of factors such as variation of RNA concentration, RT and PCR efficiency and quantitation (**Fig. 3**).
11. For the RT reaction the primer of choice may be random oligonucleotide sequences, gene specific sequences or oligo d(T). The short random oligonucleotide sequences bind randomly along the length of the RNA molecule, generating a mixture of short and long cDNA chains. These primers have been most successful for the analysis of partially degraded RNA samples, such as those commonly isolated from clinical specimens. This is most likely because they are thought to minimize the effects of mRNA secondary structure and the distance of the amplified sequences from the poly A^+ tail *(22)*. Using the 3' target gene primer will increase binding to the region of interest and assure cDNA will contain the specific target area, though some random binding is likely due to the low temperature of the RT reaction. This may be a convenient primer as it will be used in the RT and PCR. Selective amplification of mRNA can be achieved using oligo

d(T) primers, which will target the 3' poly A^+ tail found in most eukaryotic mRNAs *(23)*.

Reverse transcriptase enzyme used to form cDNA on the primed mRNA template may be avian or murine moloney derived. The most frequently used is murine moloney which is active at 37°C and rapidly inactivated by heating at 95°C for 5 min. This reverse transcriptase is a recombinant enzyme engineered to have no RNAse H activity, resulting in greater yields of cDNA. The RT reaction time may be reduced, depending on the copy number and quality of target RNA. An inhibitor of RNAses is included in the RT reaction to prevent their deleterious effect on RNA and cDNA synthesis efficiency The most frequently used are protein inhibitors isolated from human placenta which inhibit the activity of RNAses by binding them noncovalently *(24)* Dithiothreitol (1 m*M*) can be added to prevent dissociation from RNAses.

12. RT enzyme should be inactivated or used at a lower concentration than DNA polymerase (1:8) as it can inhibit the DNA polymerase activity during amplification *(25)*
13. Heating at 95°C for 5 min linearizes the cDNA and breaks the hydrogen bonding between the two strands to give two single components. Placing on ice reduces recombination of cDNA strands and prevents secondary structure reforming
14. The selection of oligonucleotide primers for amplification of target cDNA can determine its success or failure. The fundamental requirement of a primer set is that it should hybridize specifically to the sequence of interest. The length of the primer contributes to its specificity and it is advisable to choose primers between 18 and 30 bases in length. Longer primers are often chosen to improve specificity, though it is unlikely that length in excess of 30 bases will add any benefit *(23)*. Both primers should be of similar or equal length but not complementary to each other, especially at the 3' end to avoid primer–dimer formation Primer–dimers, where one primer anneals to the 3' end of the other, to produce an amplified product that will compete with the target of interest during the PCR **(Fig. 4)** Primer–dimers can be a particular problem when the target RNA copy number is low. Where possible primers should have an equal G/C and A/T concentration in a random base distribution. Primers with long stretches of polypurines or polypyrimidines should be avoided. Individual primers should not possess palidromes, to avoid secondary structures. Primers are usually selected to amplify a region of between 180 and 500 bp, though longer targets may be amplified efficiently. Amplification efficiency declines rapidly once amplified sequences are longer than 3 kb *(26)*. Localization of primers on the template can have a dramatic effect on the efficiency of amplification. Primers should be selected to avoid regions of secondary structure. Almost all preparations of RNA will contain some contaminating DNA, therefore, selection of primers separated by an intron can help identify those products from contaminating DNA (products produced from DNA would be bigger than those from RNA, reflecting amplification of the intron sequence in DNA). This is particularly important when looking for tissue–related RNA expression. Even this strategy is not infallible, the presence of pseudogenes related to the target RNA can act as a template for amplification and

generate products of the same size as the target RNA. Pseudogenes are defective genes with sequence similarities to known functional genes, but with no known biological function themselves. Construction of primers that are not complementary to known pseudogenes, or hybridization with probes specific for the target gene and not the pseudogene can help discriminate between the two Alternatively amplification from poly A^+ RNA can help resolve these problems. Primers are selected to minimize their binding to other templates in the target cDNA, if one primer binds elsewhere this will reduce the efficiency of the PCR or if both primers bind at a second site will result in an extra amplified product. To prevent this, a comparison of selected primer sequences with known gene sequences can be valuable. This can be done by checking primers against a database such as EMBL A number of primer selection programs are available which can automatically identify appropriate primers for a given target sequence using the above criteria e.g., Dieffenbach Primer Selection Programme.

15. This PCR procedure uses a hot–start, withholding the DNA polymerase from the target cDNA and primers until the reaction tube has reached 80°C or more. The reaction products are separated by a solid layer of wax from the enzyme, until the first heating step melts the wax and mixes the two aqueous layers by thermal convection. This reduces primer–dimer formation and improves amplification of low copy number targets *(27)*. A new *Taq* DNA polymerase has been produced called Ampli*Taq* GOLD (Perkin-Elmer). This enzyme is not active until heated to 95°C and so can be used to effect a "hot start" without the need to separate the reagents of the PCR reaction.

    Where a hot start PCR is not used, it is advisable to overlay reaction solutions with mineral oil (50–100 µL) or a wax pellet to reduce condensation and evaporation during amplification.

16 Enzymes with reverse transcriptase and DNA polymerase activity have been produced by a number of companies. These bifunctional enzymes (e.g., r*Tth* DNA polymerase, Perkin-Elmer) allow rapid and easy screening using RNA–PCR, and since there is only one reaction set-up, can minimize crossover contamination. However, generally the sensitivity and efficiency of these bifunctional enzymes is less than that of the hot–start two step RT-PCR described here, most likely reflecting inability to optimize conditions for RT and DNA polymerase enzymes independently.

17. Cycle conditions and number are dependent on sample quality, amplified target size, copy number and specificity of primers. Routinely DNA is denatured by heating to 90–95°C, primers anneal at 50–75°C and complementary strand synthesis occurs at 70–75°C. Denaturation to produce complete strand separation is essential for PCR, a temperature of 94°C is appropriate in most cases. The annealing temperature of primers is determined by their length and base composition. Increasing the annealing temperature will reduce nonspecific primer binding and therefore increase the specificity of amplification (**Fig. 5**), though too high and the hydrogen bonding between the primers and target sequence is disrupted. The PCR annealing temperature can equal the melting temperature or be

5°C below it. The melting temperature of a primer (temperature at which half the duplex is dissociated) can be estimated using $Tm = 2°$.(number of A+T residues) + 4° (number of G+C residues) *(28)* Extension time is dictated by the length of target being amplified, for each 1 kb the usual 1 min is certainly excessive If the target sequence is short (150–200 bp) it is possible to eliminate the extension step altogether, since the polymerase retains significant activity at lower temperatures and complete extension will occur during the transition from annealing to denaturation *(29)*. Once a method has been established it is worth evaluating shorter times.

Anywhere between 15 and 60 cycles of amplification have been described, higher cycle numbers being required for detection of low copy number targets The danger of too many cycles is detection of tissue specific genes in cells not expected to express these genes, so called "leaky" or "illegitimate" transcription *(30)*. Analyzing samples for low copy number targets, increased sensitivity and specificity can be achieved by taking an aliquot of PCR product and adding to new reaction mix to reamplify with a second set of primers which lie within the first set, this is called nested–PCR.

In theory the number of DNA fragments produced in a reaction equals 2^n, where n = cycle number i.e., 20 cycles would generate approximately 1 million fragments and 30 cycles 1 billion However, the reaction tends to reach a plateau and the number of DNA fragments produced is usually less than that predicted mathematically

18. Assure the comb is the correct length for the tank used Combs that are too long will form wells with incomplete bottoms; when samples are added they will seep between the gel and tray. The size of the comb will dictate the well size and consequently the amount of RT-PCR product loaded.
19. Bubbles can be removed using a glass pipet and bulb if this is carried out before the gel begins to set. Where a microwave is not available agarose and 1X TBE can be heated in a water bath. It is not advisable to use a hot plate as uneven heat distribution can result in explosion within the flask Agarose gels can be prepared up to 24 h in advance, once the gel is set wrap it in cling–film and place at 4°C to reduce evaporation Storage of gels for too long will result in shrinkage and cracking of the gel
20 The use of Southern blotting is valuable to confirm the identity of RT-PCR products and can increase the sensitivity of detection in some cases *(23)* Increased specificity can also be achieved by Southern blotting, where more than one RT-PCR product is produced When establishing a new RT-PCR method it is important to confirm the identity of RT-PCR products by sequence analysis

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19

Detection of Circulating Tumor Cells Using Immunobead-PCR

Jennifer E. Hardingham

1. Introduction

Many studies have shown that survival following surgery for solid tumors, such as colorectal and breast, is influenced by the extent of local invasion and the presence of distant metastases, and staging systems based on that are still the main prognostic indicator of survival. However, 20–30% of patients with early stage disease, without evidence of lymph node or distant dissemination, relapse and die within 5 yr of "curative" resection. The inference is that occult or micrometastatic disease was already present at the time of resection, or that tumor cells with metastatic potential were released into the blood stream as a result of surgery. Detection of circulating tumor cells at diagnosis in early stage patients would imply that the disease is more advanced than indicated by conventional staging. These patients might then be offered adjuvant chemotherapy or radiotherapy post surgery.

Metastasis necessarily involves transition of tumor cells through the lymph and blood circulatory systems so it is likely that tumor cells might be present in peripheral blood although at very low levels *(1)*. A very sensitive technique is therefore required for their detection and to this end we have established a two-step technique, immunobead-polymerase chain reaction (PCR), for isolating and detecting tumor cells *(2)*. This technique involves isolating epithelial cells from blood using immunomagnetic beads labeled with an epithelial specific antibody. A PCR-based analysis is performed on the lysed captured cells followed by gel electrophoresis and silver staining to identify a tumor specific mutation. In a pilot study of 27 colorectal cancer patients, using a mutation in codon 12 of K-*ras* as a tumor marker, detection of tumor cells in blood taken at

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the time of surgery was associated with significantly shortened relapse-free survival ($p = 0.0001$) *(3)*. This chapter describes the method to detect colorectal carcinoma cells in blood by immunobead-PCR using a mutation in codon 12 of K-*ras* as the tumor marker (*see* **Note 1**).

2. Materials

2.1. Labeling Immunobeads with Monoclonal Antibody

1. Immunomagnetic beads: The immunomagnetic beads, Dynabeads™ (Dynal, Oslo, Norway), are supplied by the manufacturer with rat antimouse IgG_1 (*see* **Note 2**) covalently bound to the surface. The beads are uniform polystyrene microspheres, 4.5 μm in diameter, containing ferrous and ferric oxides, thus making them paramagnetic. The beads are stable at 4°C until the expiration date on the label (12 mo or more).
2. Epithelial specific antibody: The monoclonal antibody Ber-EP4 (Dakopatts, Gestrop, Denmark) was selected as the antibody of choice.
3. Phosphate-buffered saline (PBS)-Dulbecco's calcium and magnesium free (ICN Biomedicals, Costa Mesa, CA). Dissolve packet in 1 L high purity water (HPW) (*see* **Note 3**), aliquot into 100-mL bottles and autoclave. Store at room temperature for up to 6 mo.
4. Phycoerythrin conjugated antimouse antibody F(ab') fragment (Silenus Laboratories, Hawthorn, Australia or Caltag, San Francisco, CA)

2.2. Immunobead-PCR

1. Blood samples: Collect 20 mL of peripheral blood in dipotassium EDTA both pre- and postoperatively from patients undergoing surgical resection for primary colorectal cancer.
2. PCR primers: The primer sequences were adopted from Jiang et al., *(4)* and were designed to introduce restriction enzyme recognition sites into the amplified sequence by means of single base mismatches This technique is referred to as restriction fragment length polymorphism (RFLP) analysis. The primer sequences are shown in **Table 1**.
3. Molecular biology grade water: Ultra Pure Water (UPW) (Biotech International Inc., Bentley, WA, Australia).
4. 40 m*M* deoxyribonucleoside triphosphates (dNTPs):
   a. Dissolve each 50 mg bottle of deoxynucleotide triphosphates i.e adenosine (dATP), guanosine (dGTP), cytidine (dCTP), thymidine (dTTP), (Boehringer Mannheim, Indianapolis, IN) in approx 1.5 mL of UPW, pooling each into one sterile 10-mL tube.
   b. Adjust the pH to 7 0 by the addition of sterile 1 *M* Tris base, monitoring the pH by spotting the solution onto pH paper.
   c. Make up volume to 9 mL and sterilize by filtering through a 22 μm millipore filter unit. Aliquot into 200-μL volumes and store at –20°C for up to 6 mo.
5. *Taq* polymerase and 10X PCR buffer (Boehringer Mannheim).

Table 1
Primer Sequences in 5' to 3' Orientation[a]

Primer	Sequence
K-*ras* sense (K5')	ACTGAATATAAACTTGTGGTAGTTGGA<u>C</u>CT
K-*ras* antisense (K3')	TCAAAGAATGGTCCTG<u>G</u>ACC

[a]The mismatched bases are underlined.

2.3. RFLP Analysis: Restriction Enzyme Digestion and Polyacrylamide Gel Electrophoresis

1. *Bst*N1 and 10X digestion buffer (New England Biolabs, Beverly, MA).
2. Proteinase K (Merck, Darmstadt, Germany).
3. Gelbond film (GelBond PAG, FMC Bioproducts, Rockland, MD).
4. Acrylamide: bisacrylamide 19:1 (Gradipore, Sydney, Australia).
5. 10% Ammonium persulfate.
6. TEMED, electrophoresis grade (Life Technologies, Gaithersburg, MD).
7 Electrophoresis buffer: 0.5X TBE. Make 5X TBE buffer as follows. 54 g Tris base, 27 5 g boric acid, 20 mL 0.5 *M* EDTA (pH 8.0), make up volume to 1 L with HPW, and use at 1/10 dilution.
8. 6X Gel loading buffer: 0.25 g bromophenol blue, 40 g sucrose. Dissolve in 80 mL HPW. Adjust pH to approx 10.0 with 1 *N* NaOH and make up volume to 100 mL. Store at 4°C indefinitely.
9. Vertical gel electrophoresis system (BRL model V-16, Gaithersburg, MD) with 20 cm × 16 cm plates and 0.4 μ spacers.

2.4. Silver Staining Reagents

1. Fixer solution: 10% ethanol.
2. 1% Nitric acid: dilute 14 mL 70% nitric acid (analytical reagent (AR) grade) in 1 L HPW water
3. Silver solution: 1.5 g/L $AgNO_3$ (AR), 0.05% (v/v) formaldehyde (AR) (1.5 mL/L). Formaldehyde is a flammable, poisonous liquid. Avoid contact with skin and eyes. It should be stored at room temperature since it becomes inactivated at 4°C.
4. Developer solution: 30 g/L Na_2CO_3, 0.05% (v/v) formaldehyde, 400 μg/L sodium thiosulfate. Use freshly made solution each time.
5. Stop solution· 7.5% acetic acid. Dilute glacial acetic acid (AR) in deionized water.

2.5. Special Requirements

1. 10-mL Polystyrene V-bottom tubes (sterile) for incubation of blood with labeled Dynabeads (*see* **Note 4**).
2. Magnetic tube holders for 10- and 0.5-mL tubes (Dynal MPC-6 and MPC-P-12).

3. Method

3.1. Labeling Immunobeads with Monoclonal Antibody

The choice of antibody is an important one for this application because it must have a high degree of specificity and avidity for epithelial cells and must not react with cells of hemopoietic origin. The monoclonal antibody Ber-EP4 is directed against an epitope on the protein moiety of 2 glycoproteins (34 and 39 kDa) present in the membrane and cytoplasm of most epithelial cells, the exceptions being hepatocytes, parietal cells, and apical cell layers in squamous epithelia. Importantly, Ber-EP4 is not expressed by hemopoietic cells *(5)*. Reactivity of Ber-EP4 was tested on 10 colorectal carcinoma cell lines, 4 breast cancer cell lines and 27 colon tumor samples using flow cytometry or immunohistochemistry; Ber-EP4 reacts with all cell lines and tumors tested (*2,3*, unpublished results).

1. Pipet an appropriate volume of Dynabeads, after thorough mixing, into a 1 5-mL tube. The amount of antibody required to saturate the beads has been calculated at 2 μg/mg beads, which is equivalent to 200 ng/1 × 10^6 beads Calculate the number of beads needed for the experiment based on 3 × 10^6 beads/10 mL blood sample (*see* **Note 5**). To label 24 × 10^6 beads (sufficient for eight 10-mL blood samples), add 20 μL (5 μg) of monoclonal antibody Ber-EP4 (*see* **Note 6**) and 500 μL of sterile PBS.
2. Mix on a slowly rotating mixer (e.g., Dynal Sample Mixer) for 2 h or overnight at room temperature.
3. Wash beads 1 X in PBS, resuspend in PBS and count a 1/10 dilution (e.g., 5 in 45 μL) using a hemocytometer under low power
4. Check labeling of beads by flow cytometry. Using the same dilution, add 5 μL of phycoerythrin (PE)-conjugated antimouse antibody and incubate 15–30 min on mixer. Wash 1 X in PBS, resuspend and analyze on flow cytometer. They should be >99% positive.

3.2. Immunobead-PCR

The protocol is shown schematically in **Fig. 1.**

1. Transfer blood into a 10-mL polystyrene tube and add 3 × 10^6 Ber-EP4-labeled Dynabeads (*see* **Note 7**)
2. Incubate at room temperature on a slowly rotating mixer for 4 h or overnight.
3. Epithelial cell isolation: tape the tubes to a magnetic array (*see* **Note 8**) or place in a magnetic tube holder (Dynal MPC 6) and leave for 10 min to allow beads with bound epithelial cells to settle against the magnet
4. Wash the remaining blood away 3X with PBS, leaving tubes on the magnet to retain the beads and captured cells Change gloves before next patient sample to avoid cross contamination.
5 Take tubes away from the magnet, resuspend beads in 2 mL PBS and allow tubes to stand on the magnetic array so that the beads settle in the bottom of the tube.

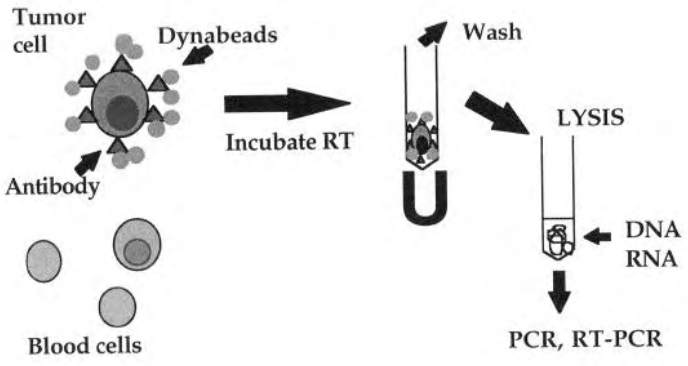

Fig. 1. Immunobead enrichment of tumor cells

6. Withdraw most of the supernatant leaving about 200 µL. Resuspend the bead pellet in this volume and pipet into a PCR tube (0.5-mL tube).
7. Finally, place the PCR tube in the Dynal MPC-P-12 magnetic tube holder. Allow beads to align on the side of the tube, then aspirate the supernatant. Resuspend the pellet of beads in 10 µL of UPW (*see* **Note 9**) and store frozen at –20°C ready for PCR analysis.
8. Set up a master PCR mix containing 1X PCR buffer (containing 1.5 m*M* $MgCl_2$), 800 µM dNTPs, 0.4 µ*M* of each primer, and 0.75 U *Taq* polymerase. Make up to 40 µL with UPW.
9. Add the mix directly to the lysed, thawed bead pellet.
10. Overlay with paraffin oil to prevent evaporation.
11. Start the amplification reaction in a thermal cycler (PTC 100, MJ Research, Watertown, MA). Begin with a 94°C initial denaturation for 5 min, then 45 cycles of 94°C 1 min, 55°C 1 min, 72°C 1 min, with a final extension of 72°C for 7 min. These PCR conditions has been optimized in order to obtain the maximum amount of product thus enhancing the detection rate of minimal numbers of mutant K-*ras* alleles. Include a positive control for the K-*ras* codon 12 mutation by using DNA from the cell line SW480. Always include a negative (no DNA) control and a normal DNA control.
12. Run the products on a 2% agarose gel and note the presence of the expected 157-bp product (*see* **Note 10**).

3.3. RFLP Analysis: Restriction Enzyme Digestion and Polyacrylamide Gel Electrophoresis

1. Restriction enzyme digestion: Digest 10 µL of product with 4 U of *Bst*N1 according to the manufacturer's instructions, i.e., 10 µL PCR product, 2 µL 10X buffer, 0.2 µL of 100X bovine serum albumin (both supplied with *Bst*N1), 4 units *Bst*N1 and UPW to 20 µL. Incubate the digest mix at 60°C for 2 hours, then add 1 µL of a 10 mg/mL proteinase K solution (in HPW) and continue incubation at 50°C for

1 h to digest proteins which interfere with the interpretation of the gel following silver staining.

2 Polyacrylamide gel electrophoresis:
   a. Use a vertical gel electrophoresis system (BRL model V-16, Gaithersburg, MD) with 20 cm × 16-cm plates and 0.4 μ spacers. Include a sheet of gel bond film to which the gel will adhere, thus making it easier to handle the gel during silver staining Treat the other glass plate with a silicone solution (e g., Rain Vision®) to prevent adherence of the gel
   b Preparation of gel solution: For a 10% polyacrylamide gel, make a total volume of 22 mL containing. 5 5 mL of 40% acrylamide/*bis*acrylamide (19 1), 2 2 mL of 5X TBE, 0.4 mL 10% ammonium persulfate, 14 mL HPW, 10 μL TEMED. Mix well and pipet into the mold Place the comb in position in the top of the gel and allow to set for at least 30 min.
   c. Load the entire digest plus 3 μL of 6X loading buffer onto the gel using flat tips (Sorenson MultiFlex®, BioScience Inc., UT). Load an undigested product control to indicate the position of the 157-bp undigested fragment on the gel. Carry out electrophoresis at 150 V for 3 h or until the bromophenol dye front is close to the bottom of the gel.

3.4. Silver Staining Protocol

To achieve high quality staining without background, chemicals must be of high purity, analytical grade and solutions prepared with deionized water. The staining trays must be cleaned of all previous silver stain by wiping out with ethanol. Gloves should be worn when handling gels to avoid staining artifacts from fingerprints.

1 Fix the gel for 10 min in 10% ethanol.
2. Pour off ethanol and add 500 mL 1% nitric acid. Leave to soak 10 min
3. Discard nitric acid solution and wash twice in deionized water.
4. Stain the gel in silver solution for 30 min at room temperature with gentle agitation.
5. Rinse briefly in deionized water then develop image in developer solution 2–10 min.
6. Stop the reaction by rinsing in 7.5% acetic acid.
7 The expected bands are: 157-bp undigested control, 143 bp if a mutation in codon 12 of K-*ras* is present (*Bst*N1 has cut only in the 3' control cutting site) and 114 bp if the sequence is normal (*Bst*N1 has cut at both sites) (*see* **Note 11** and **Fig. 2**).

4. Notes

1. This technique was only applicable to the 35% of patients in our study whose tumor carried a mutation in K-*ras*, and so we have recently developed a panel of epithelial expression markers using RT-PCR ***(6)*** so that the technique is applicable to all patients with carcinoma.
2. Dynabeads are available with immunoglobulin subclass-specific second antibodies bound to the surface, to be used with that particular subclass of primary antibody. The beads are now also available with Ber-EP4 directly bound to them (Dynal carcinoma beads).

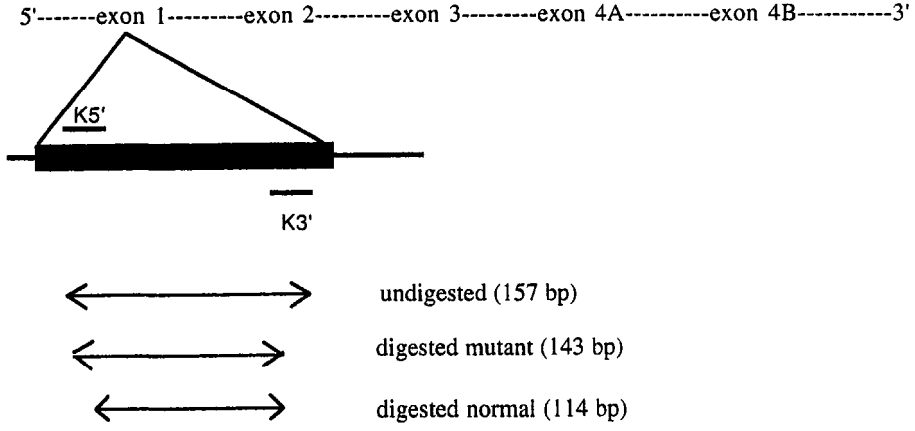

Fig 2. Diagram of the PCR product from exon 1 of the K-*ras* gene showing expected fragments following restriction enzyme digestion

3. One source of HPW is a Millipore water purification system (Milli-RO 5 plus and Milli-Q plus 185)
4 Polystyrene tubes have been found to be better for the magnetic isolation; polypropylene tubes do not work as well possibly due to electrostatic forces.
5. The number of Dynabeads required per 10 mL blood sample was derived from reconstruction experiments whereby tumor cell line cells were diluted into normal blood at a ratio of $1/10^5$ and $1/10^6$ relative to the white cell count, and immunobead-PCR performed. The intensity of the signal corresponding to the K-*ras* mutant band was read from the silver stained gel and is indicated as 3+, strong positive; 2+, intermediate; 1+ weak positive; –, negative. The results of a representative experiment are shown in **Table 2**.
6. The volume of antibody is calculated from the concentration provided in the data sheet.
7. Use plugged pipet tips to avoid carryover of positive samples.
8. A "home-made" magnetic array was used consisting of small (1 cm in diameter) cobalt samarium disc magnets mounted on a plastic tray in rows the length of a 10-mL tube.
9 For subsequent RT-PCR for which intact RNA is required, the bead pellet is resuspended in 15 µL of lysis mix containing 3 µL of 0.1 *M* DTT, 3 µL of 1% Nonidet P-40, 8 5 µL UPW, and 0.5 µL RNasin (20 U).
10 To visualize PCR products in agarose gels, add ethidium bromide (100 ng/mL) to the 0.5X TBE running buffer and view the gel on an ultraviolet light transilluminator. A 1 mg/mL stock ethidium bromide solution is made by dissolving a 10 mg tablet in 10 mL HPW. Store protected from light at room temperature. Caution: Ethidium bromide is genotoxic.
11. The sense primer was designed to contain a mismatch at position 1 of codon 11 so that C is incorporated into the sequence rather than G. This change introduces a cutting site for the restriction enzyme *Bst*N1 at CCTGG (the *Bst*N1 recognition

Table 2
Effect of Number of Immunobeads on Sensitivity of K-*ras* Immunobead-PCR

Ratio tumor cells:white cells	No of immunobeads			
	1×10^6	2×10^6	3×10^6	4×10^6
$1/10^5$	1+	2+	3+	3+
$1/10^6$	—	—	1+	1+

site is CC↓$^{T}/_{A}$GG, either T or A being acceptable). A second *Bst*N1 site was introduced via the antisense primer i.e., at CCA*G*G as a control site for *Bst*N1 digestion so that the product is cut at the 3' site regardless of whether or not the tumor has a mutation in codon 12.

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Single Cell PCR

Theory, Practice, and Clinical Applications

Ian Findlay

1. Introduction

The analysis of genetic material is fundamental to many medical and scientific applications. These include diagnosis of genetic disorders from preimplantation embryos *(1)*, prenatal diagnosis (amniocentesis and chorionic villus sampling [CVS]), forensic analysis *(2)*, analysis of pathological specimens *(3)*, paternity testing *(4)*, disease screening, (e.g., antenatal cystic fibrosis screening *(5)*, disease diagnosis, gene therapy *(6)*, formation and analysis of transgenic animals *(7)*, and investigation of oncogenes *(8)*. However, the major reason for the analysis of genetic material, particularly in medicine, is the diagnosis of genetic disorders.

Every method used for diagnostic purposes must have high reliability (defined as percentage of times a signal can be obtained or, more specifically, as the inverse of analysis failure rate), great accuracy (defined as the percentage of times the correct signal is obtained from the number of signals received), ease of performance, and, ideally, must be as informative as possible within the shortest period of time. Although a variety of techniques have been used, there is, at present, no single method that combines all these requirements, particularly for analysis at the single cell level.

One method that has been used extensively for detection or diagnosis at the single cell level, especially for single-gene defects or sex, is the polymerase chain reaction (PCR) *(9)*. PCR is particularly useful when the amount of genetic material is very limited, or samples have been degraded or fixed. The development of PCR has allowed genetic analysis from a small number of cells *(10)*. In

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recent years, the sensitivity of PCR has increased so that even single copies of genes within a single cell can be detected *(11)*. Apart from its great sensitivity, a very important advantage of PCR is that DNA fragments can be amplified within a few hours, making it possible for results to be obtained the same day. However, this high sensitivity of amplifying single cells also presents limitations and problems.

Since the major application of single cell genetic analysis has been preimplantation genetic diagnosis (PGD), the majority of this chapter will discuss this topic more specifically. However the principles of single cell analysis in PGD will be applicable to other applications, some of which will be discussed in **Subheading 1.3.**

1.1. Theory

1.1.1. Conventional PCR

Modern techniques such as PCR allow a variety of diseases to be identified in a matter of hours rather than days. However, due to problems of sensitivity and reliability at low cell numbers, most PCR analyses are still undertaken on large numbers of cells such as blood samples. Details of PCR principles and techniques are discussed in Chapter 1 and elsewhere in this volume.

1.1.2. Difficulties of Single Cell PCR

The main reasons that PCR has been found to be problematic for genetic diagnosis on low numbers of cells have been:

1. Low reliability (or high signal failure) because of poor sensitivity of many PCRs (particularly multiplex reactions) resulting from either such a low amount of signal that it cannot normally be detected, or the total amplification failure of signals,
2. Low accuracy (and thus misdiagnosis) owing to selective amplification failure (allelic dropout) of one or more signals; and
3. Misdiagnosis owing to failure to detect contamination

The difficulties of low reliability and accuracy can be countered in two main ways. First, develop a diagnostic method that is much more sensitive. Accuracy and sensitivity of PCR can be improved by using highly sensitive fluorescent PCR *(12)* that is an enhanced modification of PCR using fluorescent markers that allow easier visualization when using a DNA sequencer. This technique has already been successfully applied to genetic screening for cystic fibrosis *(5,13,14)*, Down's syndrome *(15)*, muscular dystrophy *(16,17)*, and Lesch-Nyhan syndrome *(18)*. Fluorescent PCR has been successfully adapted for use in genetic diagnosis of sex, cystic fibrosis, and also for DNA fingerprinting single cells.

Second, eliminate the problems that are causing low reliability, namely, contamination and allelic dropout.

1.1.2.1. Contamination

One of the main problems of analysis at the single-cell level is contamination that, even at normally undetectable levels, may mask or overwhelm the signal from the cell and give a false result. It is, therefore, very important that any contamination can be easily detected. Amplification of contaminating DNA in single cell genetic diagnosis could lead to serious errors when attempting specific diagnosis of single gene defects (or sex using PCR).

In PGD, one of the major applications of single cell genetic diagnosis, false negatives would not lead to a misdiagnosis but would, instead, eliminate a potentially healthy embryo from transfer. False positives, however, which might have been caused by sporadic or carryover contamination, could allow a misdiagnosed embryo to be transferred and, thus, potentially result in an affected fetus.

The extreme sensitivity of the PCR requires essential precautions to be taken to avoid amplifying extraneous DNA fragments (amplicons) from previous PCR's performed in the laboratory leading to false positive reactions and/or misdiagnosis.

Both false positive and false negative reactions can arise when a sample is contaminated. There are two main categories of contamination:

1. Naturally arising cellular DNA, e.g., from operators in the laboratory environment.
2. "PCR carryover" DNA (high concentrations of DNA from completed PCRs).

This contamination may cause incorrect signals, as the contamination may mask or overwhelm the target DNA causing misdiagnosis.

Problems surrounding PCR carryover and possible detection have been recently reviewed ***(19)*** and has been discussed in detail in Chapter 2. PCR carryover can be addressed in a number of ways: Hartley et al. have discussed the use of enzymatic methods to control carryover ***(20)***, whereas other workers ***(21)*** have described a series of steps which can be taken to reduce or eliminate this problem. These steps (and others) are listed in **Table 1**.

However, these steps used to prevent "carryover" in PCR are inadequate to completely eradicate the possibility of contamination in single-cell PCR. Despite even the best precautions, intermittent contamination can still occur, as it is very difficult, if not impossible to detect DNA carryover as these fragments are minute and are often contained as aerosol within the laboratory. It is, therefore, vitally important that reagents (including all those used in the embryology laboratory if PGD is being performed) be tested for contamination prior to every single-cell PCR. Contamination of some (but not all) PCR reagents and PCR tubes can be minimized by UV irradiation for a minimum of 15 min immediately prior to PCR.

Table 1
Measures to Reduce Contamination

a.	Physically isolating PCR preparations and products to prevent "PCR carryover", for example by preparing, running and analyzing the PCR in different laboratories.
b.	Sterilizing all reagents by autoclaving, filter sterilization or ultraviolet (UV) irradiation with due regard to their heat sensitivity.
c	Aliquoting PCR reagents to minimize the number of samplings from a single tube.
d.	Frequent changes of disposable gloves.
e.	Using tissue culture sterile techniques in a laminar flow hood and avoiding splashes when opening and closing containers containing DNA
f.	The use of specific laboratory equipment such as guarded Eppendorf tips or positive displacement pipettes to minimize the effects of aerosol contamination.
g.	Addition of DNA to previously mixed reagents as late as possible.
h.	Using negative and positive controls in each experiment.
i.	Minimizing the number of times that PCR tubes need be opened. Each opening of the tubes has the potential of contaminating the sample

The major application for single cell genetic diagnosis has been for PGD which is the diagnosis of a genetic defect before the embryo is allowed to implant into the uterus. Unfortunately, there have been many cases of single-cell misdiagnosis due to contamination reported in the literature. Although the majority have been with animal rather than human embryos, misdiagnoses have been reported in clinical cases for both sexing *(22)* and cystic fibrosis diagnosis *(23)*.

In the case of PGD, it has been found that even in vitro fertilization (IVF) water, immersion oil, growth media, and human serum can become contaminated with extraneous DNA *(24)*. To avoid the possibility of contamination from these sources, it is essential to have separate and dedicated PGD reagents in the embryology laboratory. Conventional sterile technique is not sufficient to avoid DNA contamination.

Although contamination is a major problem to be overcome in PGD, the possibility of contamination *per se* is not the main problem—but rather the detection of the contamination. For example, a contamination rate of 5% with no contamination detection system would mean that there is an identifiable risk of misdiagnosis, e.g., 2.5% (male as female and female as male). However, even if the contamination rate was 10%, the use of a contamination detection system could detect such contamination and, thus, reduce the potential misdiagnosis rate to, effectively, 0%. It is, therefore, important to not only reduce the incidence of contamination, but to also develop a system that can detect contamination wherever and whenever it occurs.

In some ways, however, the problem of misdiagnosis caused by male DNA or male cell contamination is more serious in prenatal diagnosis than in PGD. For example, when prenatal sexing of the fetus is performed for the prevention of X-linked disease, misidentification of a female fetus for a male might lead to an abortion of a healthy fetus. However in PGD, the misdiagnosis of a female fetus for a male does not cause a clinical misdiagnosis, but, instead, it would prevent the transfer of an unaffected female embryo. If the number of diagnosed female embryos during that cycle is less than two, or if the morphology of the remaining embryos does not permit the transfer of two, then the chances of pregnancy can be seriously reduced.

This problem of contamination detection in single cells has been addressed using a hemi-nested PCR method coamplifying the ΔF508 mutation and the polymorphic exon 2 of the HLA DQA1 region ***(25)***, using nested PCR and dinucleotide repeat sequences ***(26)***. Other workers ***(16)*** have used fluorescent PCR and hypervariable markers to detect and confirm carriers of Duchenne and Becker muscular dystrophy, resulting in highly informative and accurate analyses, although this has not been undertaken on single cells.

Potential problems with the diagnosis of heterozygous individuals using PCR is the possibility of either one allele failing to amplify (allelic dropout), or amplifying insufficently to be detected (preferential amplification).

1.1.2.2. Amplification Failure

The reasons for amplification failure from single cells are not known and are likely to be numerous. They may include problems with sample preparation, such as failure to transfer the cell, inefficient cell lysis, degradation or loss of the target sequence, and/or problems associated with the PCR.

Single cell amplification failure has been investigated, particularly blastomeres isolated from human cleavage stage embryos ***(27)***. It has also been reported that 5% of cleavage stage blastomeres from embryos with good morphology, and 15% with poor morphology, have no nucleus and contain anucleate cytoplasmic fragments that are often large enough to be mistaken for normal blastomeres ***(28)***. For preimplantation genetic diagnoses that depend on the presence or absence of an amplified fragment, amplification of an anucleate fragment could result in false negative results and, therefore, result in misdiagnosis.

For the selection of female embryos for transfer in couples at risk of having children with an X-linked recessive disease, a reliable method for identifying the sex is essential since males have a 50% chance of being affected. Also, in cases where diagnosis is dependent on the detection of an allele after amplification, biopsy and attempted analysis of an anucleate fragment can reduce the number of embryos that are available for transfer.

Another possible reason for PCR failure in single cells is insufficient lysis of the cell, preventing efficient amplification. The incidence of the positive and negative amplification signals varies in relation to the different cell types used *(11)*, probably because cell types, with their different structure and nature, require different lysis procedures. In the majority of unique sequences that have been examined, PCR amplification failure occurs in the region of 5–30% of single cells *(11,29–32)* depending on cell type and sequence used. In many cases, amplification failure from blastomeres from preimplantation embryos can be even higher.

1.1.2.3. Allele Dropout

Allelic dropout is allele specific PCR failure or, more specifically, the restricted amplification of an allele to such an extent that it cannot be detected even by ultrasensitive fluorescent PCR.

In PGD, allelic dropout of the Y signal for sexing (or less importantly of the ΔF508 signal for cystic fibrosis diagnosis) could lead to the transfer of a male embryo with an X-linked disorder (or less importantly of a CF heterozygote embryo). An allelic dropout rate of 25% has been demonstrated in heterozygote embryos *(33)* suggesting that some of the inaccuracy of cystic fibrosis diagnosis in single cells may be due, at least in part, to the allelic dropout of either the affected ΔF508 or the unaffected allele. However, the question of allelic dropout remains undetermined as other groups have reported no allelic dropout even in large numbers of single cell analyses *(27)*. Allelic dropout of the Y (or less importantly of the ΔF508) signal could lead to the transfer of a male embryo with an X-linked disorder (or less importantly of a CF heterozygote embryo).

1.1.2.4. Preferential Amplification

Preferential amplification is the unequal amplification of one allele compared to its sister allele or, defined more specifically, as significant hypoamplification of one allele compared to its sister allele.

An example of the difficulties presented by preferential amplification can be seen in **Figs. 1A** and **1B**, using fluorescent PCR as a comparative system. These figures show the possibility of misdiagnosis when the PCR product is below detection threshold.

1.1.3. Fluorescent PCR

Recently a modification of PCR technology using fluorescent primers and an automated DNA sequencer has improved both PCR accuracy and sensitivity *(34,35)*.

Fluorescent PCR produces a highly sensitive system (approximately 1000 times more sensitive than conventional gel analysis *(12)* allowing the detection

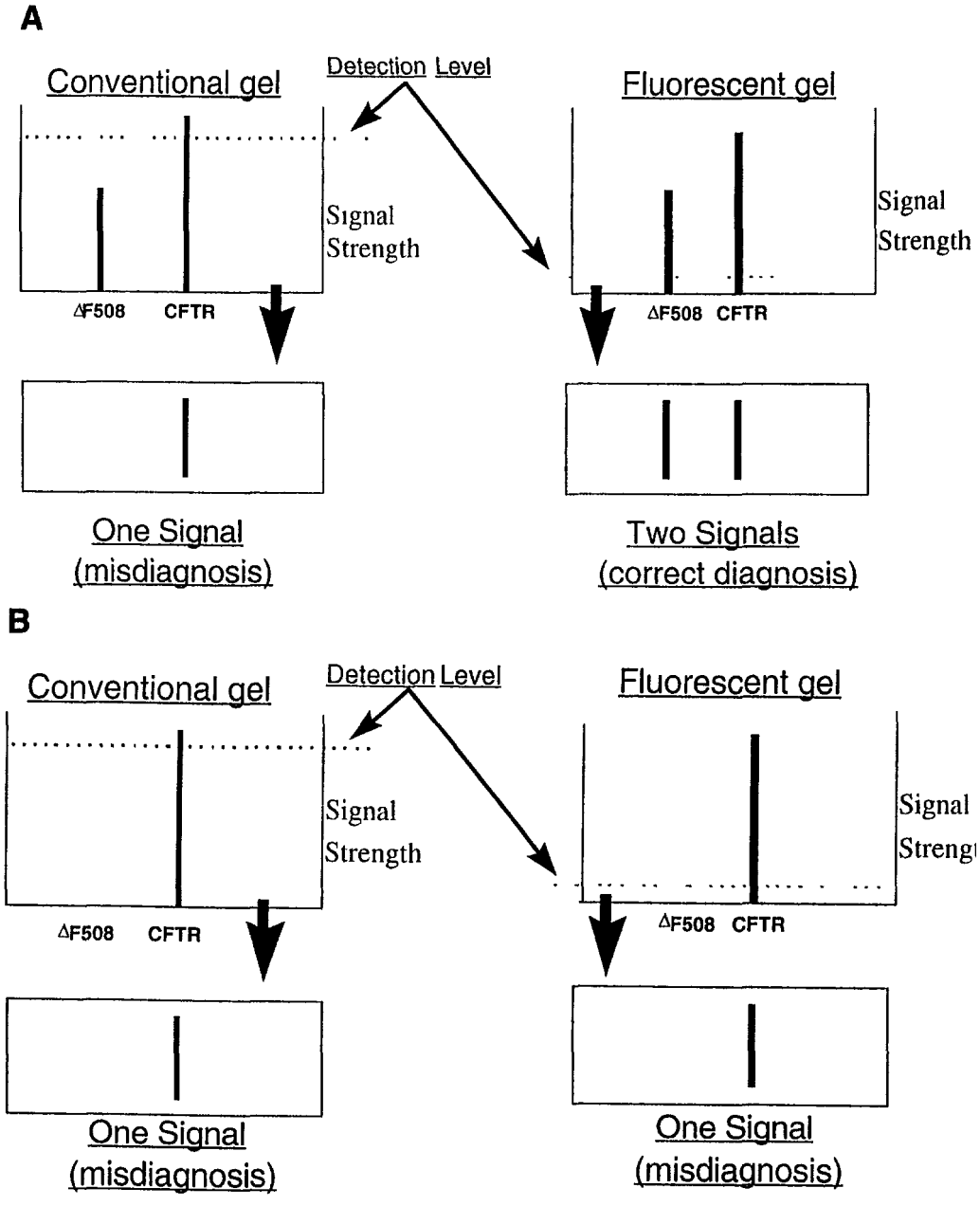

Fig. 1. **(A)** Preferential amplification in conventional and fluorescent gel analysis **(B)** Allelic dropout in conventional and fluorescent gel analysis.

of a signal far below the threshold obtained from conventional agarose or acrylamide gel analysis (**Figs. 1** and **2**). This allows highly accurate and reliable detection of signal even when the signal is very weak or many times lower than (to < 1%) that of the other allele. It is, therefore, possible to use this quantitative measurement to accurately determine the ratios of signal intensity to each other and, thus, determine the rates of allelic dropout or preferential amplification.

In practice, fluorescent PCR is very similar to conventional PCR except that each primer is tagged with a fluorescent dye. This dye can be detected at a much lower threshold level by using a photomultiplier or CCD camera and computer technology rather than conventional visualization by eye. Both conventional and fluorescent PCR methods are very similar with the exception of detection of the product.

After PCR cycling has been completed, the PCR product must be visualized to determine its quantity and molecular size. With conventional PCR, this is commonly performed using size-separation of products on agarose or acrylamide gels. The product is loaded onto the gels and after an electric current is applied, the product migrates down the gel. This method of viewing using conventional PCR is reliable and accurate only when there is a large amount of product of a specific known size. As smaller products migrate faster down the gel, it is possible, using an appropriate size marker, to determine the relative size of the product. It may often be difficult to discriminate product signals from faint inconsistencies within the gel, particularly when product yield is low. It may also be difficult to accurately size the product because of migration variation, as the size marker needs to be extrapolated across the gel. Although product molecular size can be determined using appropriate size-standard markers, this may be difficult if the product yield is low, producing only a faint signal. Also, the migration rate across the gel may differ, rendering the standard values inaccurate. These methods may be particularly inaccurate when distinguishing between small allelic differences such as one or two base pairs.

The fluorescent PCR technique is made more sensitive by using an ultrasensitive system fluorescent DNA sequencer (such as the 373A DNA sequencer with the 362 Genescanner software [Applied Biosystems, Warrington, UK]) commonly known as a Genescanner, to separate, detect, and analyze the fluorescently labeled PCR products.

The Genescanner contains a laser that scans the acrylamide gel every few seconds. As the fluorescent product passes across the laser's path, it absorbs light at a particular wavelength emitting it at a different wavelength then which passes through a filter and photomultiplier before being analyzed by a computer. Computer analysis of these signals enables the different primer products to be easily differentiated by their distinct emission wavelengths, and their

quantity determined by the relative intensities of their fluorescence. The computer analyzes these signals and constructs a representation of the gel from which electrophoretograms are generated allowing quantification.

The allele sizes of the products can be determined within 1 bp, both accurately and consistently, by including fluorescent size standards within each lane containing PCR product. As these size standards are internal to each lane and in each individual reaction, variations in product migration and lane leakage do not detract from accurate sizing. Conventional analysis methods, which depend on extrapolating size standards across the gel, are less accurate particularly when distinguishing between small allelic differences such as one or two base pairs. The internal standards also act as a positive control step for the Genescanner in case of amplification failure. The higher sensitivity of fluorescent PCR up to 1000-fold *(12)* allows visualization of levels of product below the visualization thresholds of conventional agarose/acrylamide analysis (**Figs. 1A** and **1B**).

Signal detection can occur with fewer PCR cycles than with conventional PCR (**Fig. 2**). For example, a signal can be detected after 36 cycles using fluorescent PCR, whereas, conventional PCR would require 71 cycles due to PCR plateau. This results in the time required for the PCR being dramatically reduced from 5–6 h to approx 2 h.

1.2. Practice

1.2.1. Sexing

One of the main applications of single cell sexing has been for PGD of sex-linked disorders either in domestic species (particularly cattle) or in humans. For sex diagnosis, the problem of misdiagnosis owing to amplification failure has been previously addressed *(36)* using two PCR probes: one for the Y-repeat sequence and a second probe for a gene (usually a housekeeper gene) to verify the presence of cells.

This approach provides four possible results:

1. Male cell = two unambiguous bands;
2. Female cell = single unambiguous band
3. Failed PCR = no bands; and
4. An ambiguous signal where there is a single Y-band only or the bands cannot be clearly distinguished, must be treated as a PCR failure.

This approach, although significantly reducing the false negative problem, does not exclude the chance of an unrecognized false negative result *(37)* and still has two disadvantages:

1. The PCR reaction must be optimized to work reliably at the single cell level using two potentially different primer sets that may have very different PCR requirements, thereby, potentially compromizing both reactions.

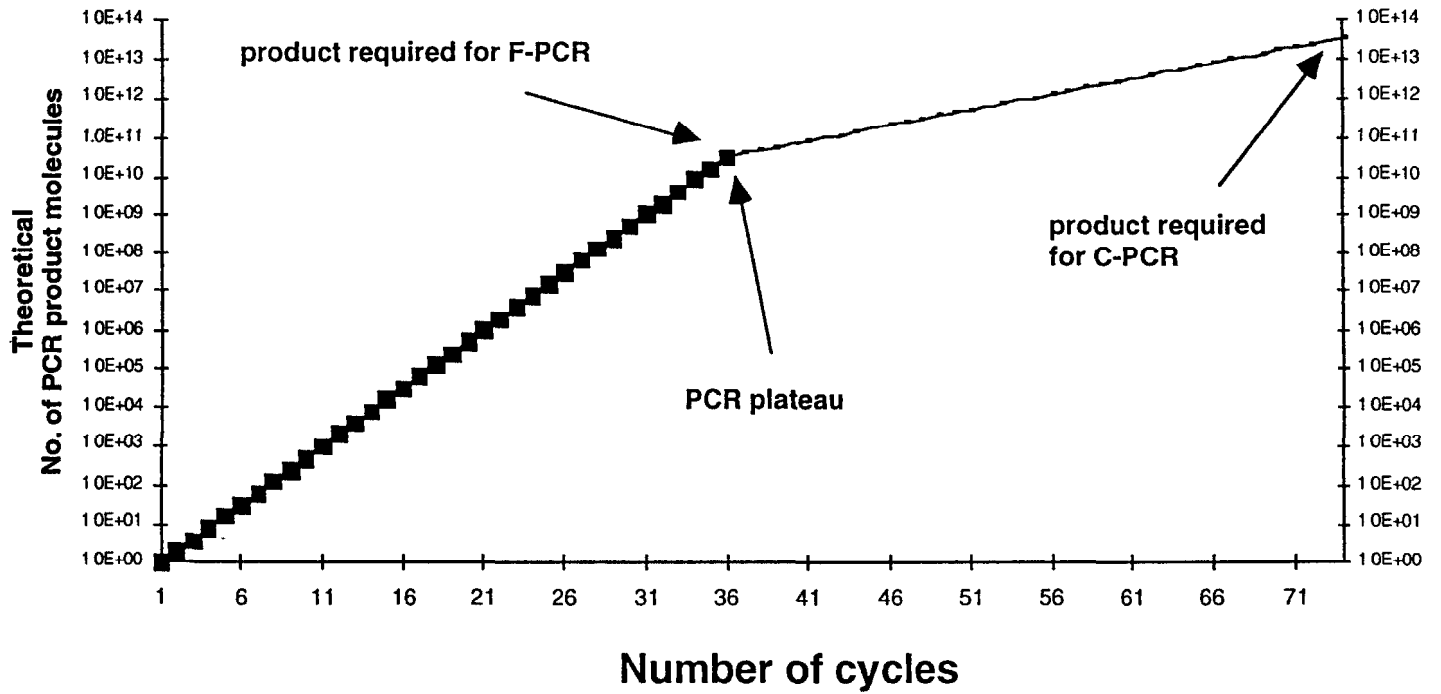

Fig. 2. Theoretical relative sensitivities of fluorescent and conventional PCR. C-PCR, conventional PCR; F-PCR, fluorescent PCR. Solid line, C-PCR; ■ F-PCR.

2. Difficulties of quantification mean that this approach cannot distinguish between sex-chromosome aneuploidies such as XX, XXX, XO or between XY, XYY, XXY.

Although there is an identifiable risk of misdiagnosis of these aneuploidies, this risk is relatively minor as:

1. XXX and XYY show no known abnormal phenotype;
2 XXY (Klinefelters syndrome) risk is 1 per 1000 liveborns and would not be transferred as the object of preimplantation sexing is to prevent the transfer of male embryos and, therefore, such embryos would not be transferred;
3. XO (Turners syndrome) has a low live-birth incidence of 1 in 12,500 and since there is a 50% chance that the affected X-chromosome will be inherited, the true risk of an affected child is 1 in 25,000.

Previous work using conventional PCR has demonstrated that the sex of a single somatic cell can be determined using primers for the amelogenin gene *(38)*. This gene has also been used for sexing of forensic samples *(39)* and has the advantage that, although present on both the X and Y chromosomes, the gene signals are 6 bp different (the X-chromosome being the smaller), allowing rapid identification of each chromosome *(40)*. This inbuilt X-linked internal control gave a correct diagnosis of 98% in a series of single somatic cells using conventional PCR *(38)* and 97% using fluorescent PCR *(31)*. Technical aspects are also much simpler as the need to optimize for nested primers is eliminated. In addition, contamination potential is reduced since reaction tubes are not opened to introduce a nested primer set.

In diploid cells, a single PCR reaction can detect both copies of the gene. If there are two signals (1 for X, 1 for Y) the cell is male, if there is only a single signal (X) then the cell has a double copy of X and is female *(2,41)*.

The amelogenin gene has the following advantages for sex identification of single cells:

1. Easier interpretation, as a single gene is used to determine the presence of both the Y- and the X-chromosomes allowing a more accurate diagnosis to be made,
2. A built-in control for the presence of cells,
3. As the system does not require nested primers, optimization of only one pair of primers is needed This feature results in a much simpler system to implement.

1.2.2. Detection of Trisomies

The detection of trisomies (particularly Trisomy 21) is a major reason for prenatal diagnosis. Most trisomies are detected by karyotype analysis, although this often takes several weeks resulting in diagnosis at 14–20 wk gestation or later. This results in the mother having a long wait for reassurance for a healthy pregnancy or the prospect of a late termination in the second trimester which is

both emotionally and physically traumatic. Ideally trisomy diagnosis would be undertaken within a single day to obviate such anxieties.

Trisomies can be detected within 24 hr using quantitative PCR of a D21 microsatellite, although very large numbers of cells are required which limits use in prenatal diagnosis *(42)*.

However, preliminary results *(43)* have demonstrated that a similar system, using the enhanced sensitivity and quantitative nature of fluorescent PCR, allows rapid (6 h), reliable (86%, 194/225) and accurate (90%, 174/194) detection of D21 microsatellites in as few as 2–5 cells*. This system, once optimized, could provide highly reliable and accurate trisomy diagnosis within a single day, thus, obviating patient anxiety. This technique also has specific potential in early prenatal diagnosis such as fetal cells from the maternal circulation (**Subheading 1.3.2.**) or from coelocentesis cells (**Subheading 1.3.3.**).

1.2.3. Single Gene Defects

The diagnosis of single-gene defects, however, has in some ways been more difficult than sex-linked disorders as only a single technique (PCR) can currently be used to detect these defects.

For PGD, PCR has been successfully used to diagnose the cystic fibrosis status *(24,23,45)* in human embryos, and sickle-cell anemia in both human *(30)* and mouse embryos *(46)*. However, both reliability and accuracy remain suboptimal. Data from the diagnosis of single-gene defects for PGD of human embryos has recently been compiled *(47)*.

Although a variety of single-gene defects have been identified as suitable candidates for PGD, most clinical cases have been undertaken for cystic fibrosis (CF), the most common lethal autosomal defect in caucasian populations. Although CF screening has concentrated only on the ΔF508 CF mutation because of its high incidence, several hundred other CF mutations have been diagnosed.

DNA diagnosis in families segregating for the common mutation ΔF508, or one of the less common mutations (e.g., G542X, G551D, etc.) is relatively straightforward as many DNA laboratories now test for these mutations using commercially available kits. However, approximately 27% of Caucasian families carry a rare CF gene mutation. Screening for the remaining known mutations is a very difficult task since several hundred CF gene mutations have already been discovered.

There are several techniques still under development for rapid and inexpensive detection of numerous alleles including the oligo-ligase chain reaction

*Quantitative fluorescent PCR has also recently been used to diagnose Trisomy 21, Trisomy 18, and Trisomy 13. *(60)*.

(often referred to as oligo ligation assay) and reverse dot hybridization. Oligoligase chain reaction utilizes two oligonucleotides chosen from sequences immediately flanking a mutation. These nucleotides are joined by DNA ligase if the mutation is present, allowing detection ***(48)***. Although this procedure is not in common use at the present time, this method, that may detect more than 30 mutations, is currently undergoing several research trials to assess suitability for clinical application.

The reverse dot hybridization procedure has been successful in human haplotyping and appears suitable to disorders with many mutant alleles (*see* Chapter 1). The amplified DNA is denatured and hybridized to oligonucleotides corresponding to mutant or normal sequences affixed to a nitrocellulose strip. Unannealed DNA is removed by washing. To detect the presence of the biotin-labeled DNA, avidin bound to horseradish peroxidase is incubated with the nitro-cellulose strip. A color change indicates the genotype of the PCR-amplified DNA, Nitrocellulose strips which could detect 15 CF gene mutations or more are currently under development. Despite these technical advances in mutation detection, it is unlikely that direct detection of the mutations in all CF patients will be possible.

If known CF mutations cannot be detected in both members of the at-risk couple, linked DNA restriction fragment length polymorphism (RFLP) markers linked to the CF gene may be used to identify the CF bearing chromosome. There is now a large number of closely linked RFLP markers in use which can be used in almost all families, particularly if a previous index-linked child is available for testing. The use of two markers flanking the CF gene minimizes the risk of misdiagnosis which may occur due to recombination events. Two approaches can be used: the first is to search for the presence or absence of a recognition site and then to run both cut and uncut products on a gel. However this technique has been criticized as incomplete sample digestion may confuse interpretation. The second approach is to use allele specific hybridization to PCR amplified material.

One particular RFLP in intron 17b is highly polymorphic. This has been used to detect more than 24 alleles with more than 95% of individuals heterozygous. This RFLP can be detected simply by using labeled primers and electrophoresis. Since this is an intragenic RFLP with high informativeness, it may become the method of choice for testing of CF using indirect means. However, when there is no DNA from a previously affected child available, the use of linked markers becomes very difficult indeed.

PCR amplified DNA can also be directly sequenced to determine whether a particular mutation occurs. This approach enables the detection of almost any mutation within a particular region, and is especially useful where clusters of mutations are known. However, sequencing is very expensive and often

technically difficult and is, therefore, not suitable for routine mutation analysis. Several methods can rapidly detect sequence alterations including denaturing-gradient gel electrophoresis (DGGE), single-strand conformation polymorphisms (SSCPs), and chemical cleavage. Each of these methods have been successfully used to search for mutations. However, DGGE appears to be the most sensitive of the three techniques. DGGE takes advantage of changes in characteristics of double stranded DNA caused by single base changes, these changes cause the DNA to migrate down a gel at different rates and, thus, allows detection. The use of DGGE to screen regions where multiple mutations occur would eliminate need for multiple restriction digestions.

Unfortunately, these techniques for detecting multiple CF mutations have yet to be applied at the single cell level for clinical applications and, therefore, at present, it is not possible to detect these mutations in preimplantation embryos.

In addition, potential problems with the diagnosis of heterozygous individuals are allelic dropout and preferential amplification. Allelic dropout of the Y in sexing (or less importantly of the ΔF508 in CF diagnosis) signal could lead to the transfer of a male embryo with an X-linked disorder (or less importantly of a CF carrier embryo).

1.2.4. DNA Fingerprinting

Hypervariable markers such as short-tandem repeat sequences (STRs)/microsatellites are widely used for gene mapping, linkage analysis, forensic identification, and genetic diagnosis ***(34,35,49)***, and are rapidly becoming the method of choice to distinguish individuals ***(50)***. With matching probabilities of between 10^{-7} and 10^{-8}, DNA fingerprinting by microsatellite typing can give a highly reliable and highly accurate genetic fingerprint for identification purposes ***(50)***. These techniques are now used on a routine basis for forensic identification of individuals and samples left at a crime scene ***(51)***.

Previous disadvantages of using microsatellites such as long run times on acrylamide gels, radioactive labeling, the accuracy of determining allele size owing to stutter bands and the absence of internal lane standards have been overcome by using the 373A DNA sequencer with gene scanner software (Applied Biosystems, Warrington, UK). Automated sizing to 1–2 base accuracy is now obtainable in 3–5 h on 12 cm gels.

The main source of markers has been tetrameric short tandem repeats (STRs) which are highly variable and are also easy to amplify. These STRs can be multiplexed in a single reaction which produces a STR profile. DNA fingerprinting using these polymorphic markers has been used for a variety of purposes; paternity testing, gene mapping, linkage analysis, forensic identification, and studies of genetic events such as loss of heterozygosity in cancer detection ***(52)***.

1.3. Practical Applications

1.3.1. Preimplantation Genetic Diagnosis

A variety of techniques have been used for single-cell genetic diagnosis. In general, PCR has been used most often, particularly as other techniques are unable to rapidly detect single-gene defects especially from small amounts of material. However, for single-cell sexing, PCR has been currently abandoned in place of fluorescent *in situ* hybridization (FISH) due to signal failure (causing misdiagnosis) of multiple PCR of X and Y regions. The main reason for the high signal failure of many PCRs, particularly multiplex reactions, is amplification failure (allelic dropout) of one or more signals, which can be reduced using fluorescent PCR ***(31)***.

Fluorescent PCR can be used for both sex and single-gene defect diagnosis, whereas FISH is used for sexing and conventional PCR is used for single-gene defect diagnosis. PCR can also often be performed on the 10–20% of blastomeres that lyse during biopsy. If lysis occurs, FISH cannot be performed and another blastomere must be biopsied. This additional biopsy involves risking the integrity of the embryo again and increases the possibility of damaging the embryo. As usually only a single blastomere is biopsied, this problem may be overcome by encouraging the blastomere to replicate in vitro, but at present this has met with only limited success in humans ***(53,54)***.

Instead of performing sexing and embryo transfer the same day, an alternative option that would permit more time for genetic testing would involve cryopreservation of the biopsied embryos and transfer in a later cycle. Biopsied 4-cell mouse embryos have been cryopreserved with an ultra-rapid protocol and have developed to term after thawing and transfer ***(55)***. However, the slit left in the zona pellucida after the biopsy might increase the possibility of the embryo being damaged, as the zona is thought to protect embryos from damage caused by ice crystal formation in the medium. Although pregnancy rates after transfer of frozen embryos is equal to those without freezing, around 20% of the frozen embryos are lost in the freezing process. Until the feasibility of cryopreserving biopsied human embryos is optimized, the best alternative method was considered to perform the diagnosis and transfer during the same day that in vitro fertilization (IVF) transfers are routinely performed.

1.3.2. Fetal Cells in Maternal Circulation

The possibilities of enriching, detecting, isolating, and analyzing circulating fetal cells in the maternal circulation are addressed elsewhere in Chapters 21 and 22.

Recently, sex, Duchenne muscular dystrophy (DMD), and rhesus type have been determined at the single cell level (discussed in further detail in Chapter 22) in fetal nucleated erythrocytes obtained from the maternal circulation using

primer extension preamplification (PEP) and nested PCR *(56,57)*. However, one of the major difficulties of using fetal cells is that it is difficult to determine if the isolated cell is indeed fetal rather than maternal, particularly if the fetus is female. However, the DNA fingerprinting methods previously discussed in this chapter may be applied to such cells to determine origin (fetal or maternal) in the same way as DNA fingerprinting of a single PGD sample can determine if the sample is embryonic or contaminated by maternal or other cells. **Fig. 3** demonstrates how such a system could be used.

1.3.3. Prenatal Diagnosis (Coelocentesis)

Prenatal diagnosis is currently undertaken to detect a variety of genetic disorders such as chromosomal aneuploidies and single-gene defects at a relatively late stage of pregnancy (11–18 weeks) either by amniocentesis or chorionic villus sampling (CVS). Although prenatal diagnosis is highly accurate (> 99%), there are significant disadvantages including an approx 1% risk of miscarriage, and often significant delay (several weeks for cell culture and karyotyping) before the results of the tests are known. Although CVS can be undertaken earlier, there has been some recent concern over the risks of miscarriage and limb defects that has restricted the use of CVS to 11 wk and beyond *(58)*.

Recently, an alternative potential method of obtaining fetal cells at a much earlier stage of development has been devised. This technique, termed coelocentesis, obtains cells from the coelomic cavity at 7–12 wk gestation *(59)*. Recently, it has been established *(14)* that these coelomic cells can be used in a highly sensitive fluorescent PCR system to provide a rapid (4–5 h), reliable and accurate multiple genetic diagnoses (sexing and single-gene diagnosis) as well as simultaneous DNA fingerprinting to ensure that contamination has not occurred (**Fig. 4**).

This earlier method of prenatal diagnosis would be very valuable as it may overcome some problems of later conventional prenatal diagnosis and allow reassurance/treatment to be offered much earlier. Successful application of these techniques may supersede alternative methods of prenatal diagnosis. Although these techniques appear very promising, extensive clinical trials must be undertaken to determine the safety of coelocentesis, diagnostic reliability, and accuracy in a clinical setting.

These techniques can also be applied to conventional CVS and amniocentesis allowing rapid multiple diagnoses to be made.

1.3.4. Forensics

As DNA fingerprinting requires relatively large amounts of DNA (1 ng/ approx 300 cells and often more for reliable typing), there are many occasions where fingerprinting cannot be undertaken because of an insufficient sample.

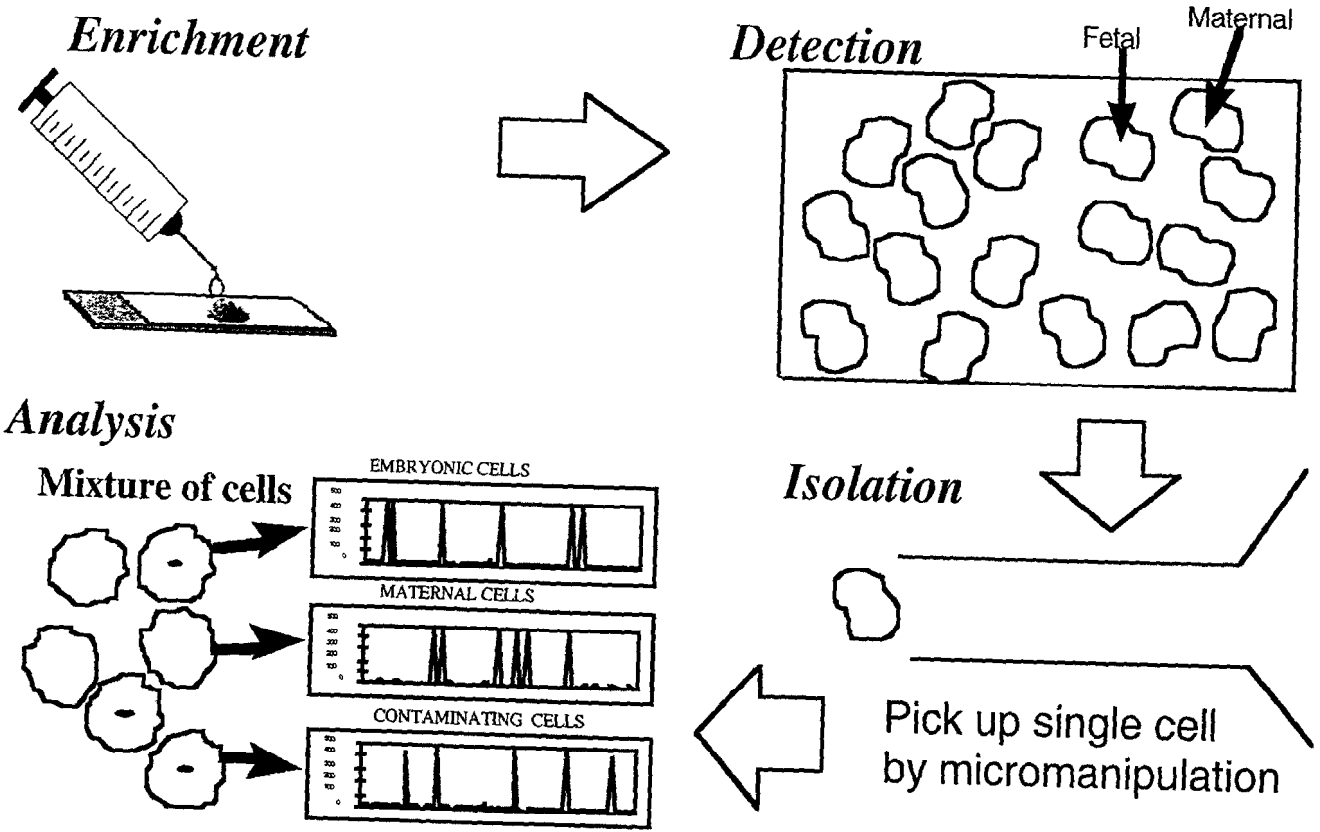

Fig. 3. Isolation of fetal cells in maternal circulation.

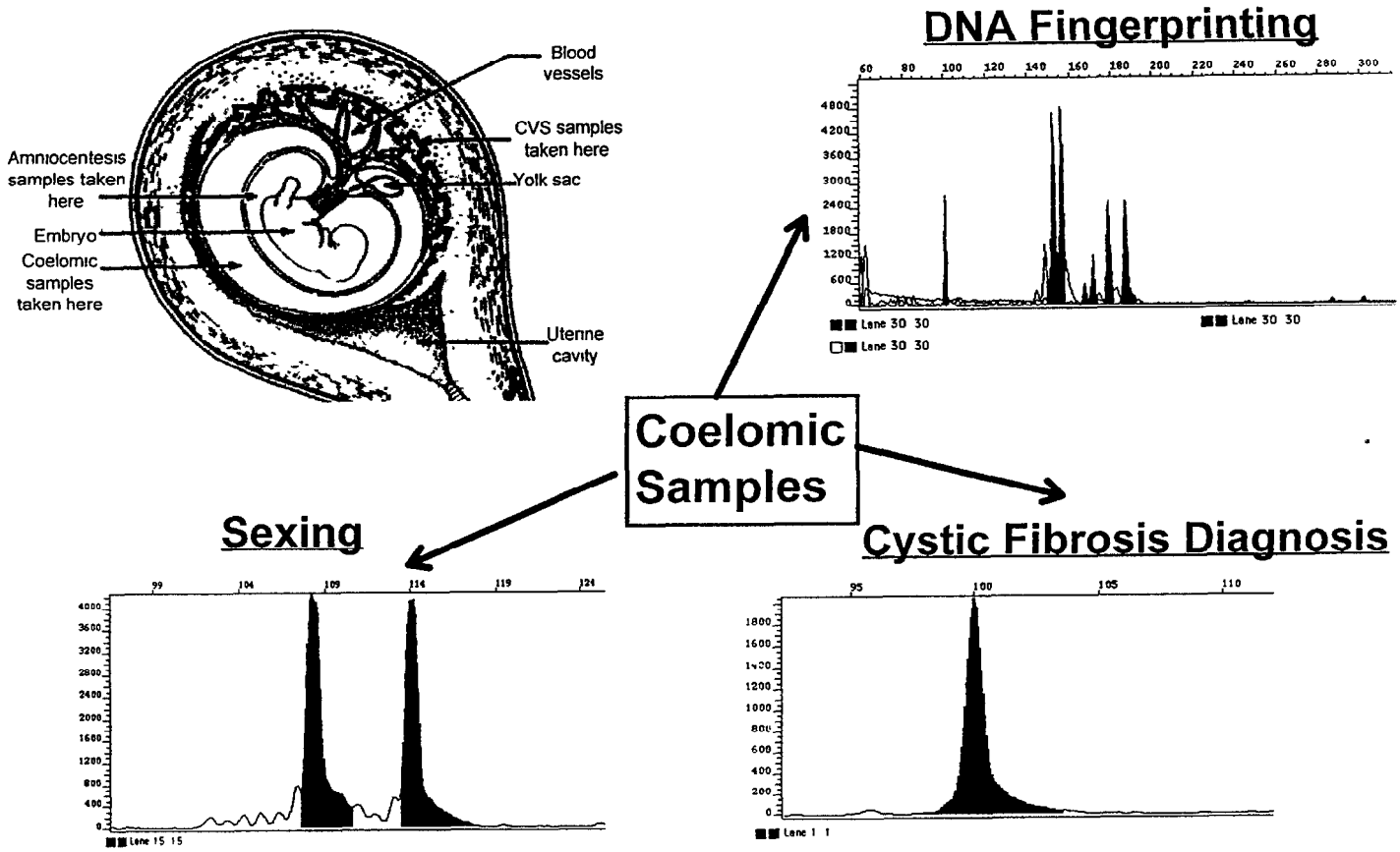

Fig. 4. Prenatal diagnosis by coelocentesis.

1.3.4.1. Low Numbers of Cells

One exciting application of DNA fingerprinting of single cells in forensic science would be analyzing difficult samples such as cells left at a crime scene e.g., from within a physical fingerprint or from dandruff, therefore allowing identification of an individual from a single cell even if the fingerprint is smudged or only partially present. If there are many cells within the sample, it may also be possible to subtract known DNA profiles leaving the suspect DNA profile. This may also allow identification of an individual even if another fingerprint was applied over the original fingerprint.

An additional problem is that forensic samples for DNA analysis can become easily contaminated by other cells. Currently, if a large sample is analyzed, the DNA from these contaminating cells may be amplified and provide additional peaks which may make the DNA fingerprint difficult to interpret and result in lower confidence in the analysis. However, if such large samples were dissected into smaller pieces and analyzed separately, each result could be compared with each other and erroneous samples eliminated. Multiple results would also be useful particularly if a single sample failed to amplify—allowing more than one bite at this particular cherry.

Recent results have obtained full and partial DNA fingerprints in 96% and 71%, respectively, in single cells *(13)*. However, although these results are encouraging, this system must be optimized to increase reliability and accuracy (by minimizing allele dropout and amplification failure) before single-cell DNA fingerprinting can be robust enough to allow the source of a single-cell to be routinely established.

1.3.4.2. DNA Fingerprinting of Single Sperm

One intriguing application of single-cell PCR is the DNA fingerprinting of single spermatozoa, particularly for forensic applications. One example is in rape cases. Although identification of an individual can be performed using DNA fingerprinting from a single semen sample, in many cases semen samples are obtained which are either mixed with other semen or are heavily mixed with vaginal or cervical cells from the female. In these cases, forensic identification of the assailant is very difficult.

In these cases, individual spermatozoa could be isolated from the sample, washed free of any cell fragments that may be present, and subjected to single cell DNA fingerprinting. This approach is demonstrated in **Fig. 5.** If successful, this would allow a single individual sperm to identify the assailant. Although very promising, this approach is limited by the haploid nature of spermatozoa that would result in a lower specificity as each microsatellite would be homozygous at each locus. However, the successful application of

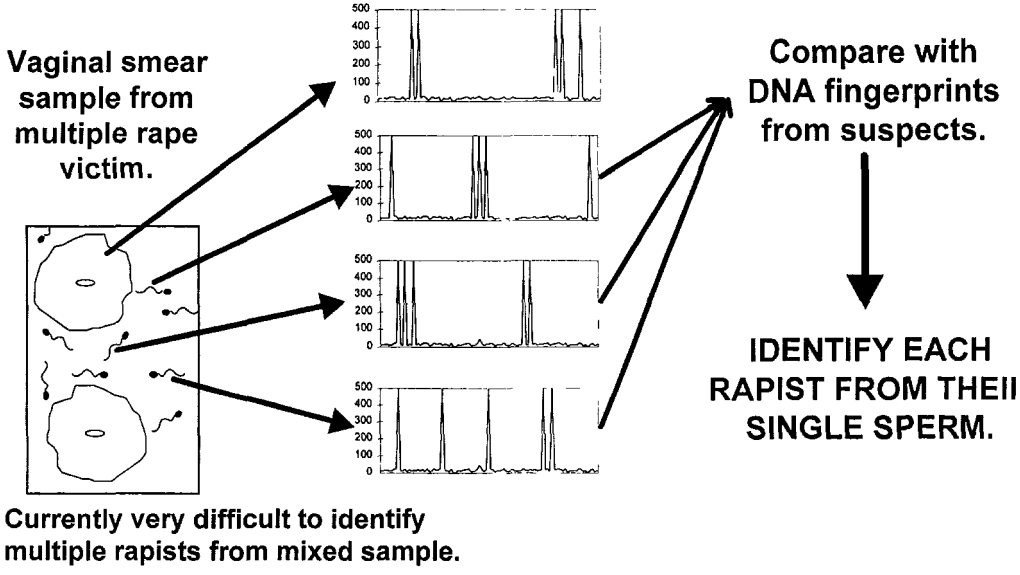

Fig. 5. Single cell forensic identificaiton by DNA fingerprinting.

these tests would, at the very least, be additional supporting evidence or would allow potential suspects to be rapidly eliminated.

2. Materials

2.1. Single Cell PCR

The following conditions require optimization for each set of primers to maximize product yield. These values are those typically used for a 50 µL reaction volume, but individual adjustment of each parameter is often necessary:

1. Target DNA: DNA can be amplified from virtually any source of genetic material, (e.g., buccal cells from mouthwashes, blood, cell lines, human hair, sperm cells, embryonic cells, forensic samples, single cells, archaeological specimens, or from poor quality/crude samples such as pathological material). The limiting factor is that the DNA sequence must be known so that the appropriate complementary primers can be synthesized.
2. Primers: Typical primer concentration is between 0.1–0.5 µ*M* per reaction (*see* **Note 1**).
3 dNTP: (MBI, Lithunia) A typical 50 µL PCR contains 200 µmol of each dNTP. Primers sequences are listed in **Table 2**.
4. *Taq* polymerase: Thermoprime Plus, (ABT), usually 1–1.5 U of *Taq* are used for each 50 µL reaction (*see* **Note 2**).
5. PCR buffer: Reaction buffer IV, (Advanced Biotechnologies, Epsom,UK (ABT)

6. Pasteur pipette
7. Eppendorf tubes.
8. Thermal cycler (DNA engine, Genetic Research Instruments, Essex, UK).

2.2. Analysis of product

1. DNA sequencer such as the ABI 373 sequencer (Applied Biosystems, Warrington, UK).
2. Acrylamide.
3. Gel plates.
4. Nonfluorescent detergent.
5. Spacers.
6. Plastic clips.
7. Sequencing gel mix (Applied Biosystems, ABI).
8. Ammonium persulphate solution: 0.1 g ammonium persulphate dissolved in 1 mL distilled water.
9. TEMED.
10. Gel comb.
11 Ethanol.
12. TBE buffer: 0.09 *M* Tris-borate, 0 002 *M* EDTA, pH 8.0.
13. GeneScan standard (Applied Biosystems)
14. Formamide.

3. Methods

3.1. Single Cell PCR

1 Transfer cell sample, using a pulled Pasteur pipet and a minimum volume of fluid, to a sterile 0.5-mL Eppendorf tube and freeze at –20°C for at least 2 h until use. Alternatively, if time is of the essence and it is necessary to run the samples as quickly as possible, samples may be frozen at –70°C for 15 min.
2. Program the thermal cycler/PCR machine (*see* **Note 3**). The program in **Table 3** is suitable for sexing using amelogenin primers.
3. If oligonucleotide primers are stored in ammonia, desiccate for 20 min. For sexing, use primer concentrations of 0.05 μ*M* for both amelogenin primers for each reaction.
4. Make master mix. PCRs are performed in 25 μL reactions using master mix comprising 1X PCR buffer (Reaction buffer IV, Advanced Biotechnologies Ltd, UK (ABT), 200 μ*M* of each dNTP (MBI), 1.5 μ*M* $MgCl_2$, 0.6 U of *Taq* polymerase (Thermoprime Plus, [ABT]). Primer concentrations were 0.8 μ*M* for the amelogenin primers; 0.25 μ*M* for the CF primers; and 0.2 μ*M* D21, 0.05 μ*M* D18, 0.25 μ*M* VWA, 0.22 μ*M* THO, 0.06 μ*M* FGA, and 0.25 μ*M* D6 for the microsatellite primers.
5. Add 25 μL of master mix to each tube containing the cell sample.
6. Overlay the reaction mixture with 40 μL of mineral oil to prevent evaporation.
7. Mix the solution well. Load tubes into PCR machine. Ensure that all tubes are firmly within their holes and start the program.

Table 2
Primer Sequences

Locus	Location	GenBank Accession number	Primer name	Primer sequence	D
D21S11	21	M84567	D21S11-1	5'-ATA TGT GAG TCA ATT CCC CAA G-3'	F/
			D21S11-2	5'-TGT ATT AGT CAA TGT TCT CCA G-3'	
D18S51	18q21 3	L18333	D18S51-1	5'-CAA ACC CGA CTA CCA GCA AC-3'	F/
			D18S51-2	5'-GAG CCA TGT TCA TGC CAC TG-3'	
HUMWVFA31/A	12p12-pter	M25858	VWA/A1	5'-CCC TAG TGG ATG ATA AGA ATA ATC AGT ATG-3'	H
			VWA2	5'-GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG-3'	
HUMTHO1	11p15-15.5	D00269	THO1	5'-GTG GGC TGA AAA GCT CCC GAT TAT-3'	F/
			THO2	5'-GTG ATT CCC ATT GGC CTG TTC CTC-3'	
D8S1179 (D6S502)	8		D8S1179-1	5'-TTT TTG TAT TTC ATG TGT ACA TTC G-3'	Tl
			D8S1179-2	5'-CGT AGC TAT AAT TAG TTC ATT TTC-3'	
HUMFIBRA	4q28	M64982	FGA	5'-GCC CCA TAG GTT TTG AAC TCA-3'	H
			FGA2	5'-TGA TTT GTC TGT AAT TGC CAG C-3'	
HUMAMGXA and HUMAMGY	Xp22 1-p22 3 Yp11 2	M86932 M86933	AMEL-A AMEL-B	5'-CCC TGG GCT CTG TAA AGA ATA GTG-3' 5'-ATC AGA GCT TAA ACT GGG AAG CTG-3'	F/
CF and ΔF508	7qCFTR		CF1	5'-GTT TTC CTG GAT TAT GCC TGG GCA-3'	H
			CF2	5'-GTT GGC ATG CTT TGA TGA CGC TTC-3'	

Table 3
PCR Thermal Profile for Sexing Using Amelogenin Primers

Step	Duration	Temperature, °C
Initial denaturation	300 s	95
Denaturation	45 s	94
Annealing	45 s	60
Extension	45 s	72
Steps 2–4 were repeated 39 more times		
Final extension	10 min	72
Cooling	indefinite hold	4

8 Denature at 95°C for 5 min then cycle the reaction for 40 cycles of 94°C, 60°C, 72°C for 45 s at each temperature followed by a 10 min at 72°C before holding at 4°C on a thermal cycler (DNA engine, Genetic Research Instruments, Essex, UK). The program runs for approx 3 h
9. Whilst the PCR's are running, preparations for analysing the product can be made.

3.2. Analysis of Product

The analysis of the fluorescent product can be analyzed on a DNA sequencer such as the ABI 373 sequencer (Applied Biosystems [ABI]). Using the ABI 373 sequencer, this procedure can be divided into six stages:

1. Making the gel.
2 Calibration of Genescanner.
3. Sample Preparation.
4. Computer preparation.
5. Loading the samples.
6. Gel analysis.

3.2.1. Making the Gel

1. The gel must be made at least 1–2 h before, to allow the gel to set. Acrylamide is toxic so gloves must be worn.
2 It is vitally important that the gel plates are thoroughly clean. If plates are dirty, bubbles will form preventing product migration and product will be lost.
3. Thoroughly wash two 12-cm glass plates (ABI) and soak with a sachet of nonfluorescent detergent. Thoroughly wash both sides of each plate. Remove plates from detergent and thoroughly wash the plates with running water. Place plates up to dry.
4. Dry plates and spacers thoroughly. Place gel plate onto a small box onto bench (making sure that the outside end of the plate faces downward). Place spacers at left and right edges, then add the second plate to top (make sure that outside end faces upwards). Add 4–6 plastic clips to plates to keep the plates in position.

5 Make up acrylamide gel solution. Add 30 mL of sequencing gel mix (Severn Biotech ABI) to a squeezy bottle. Add 1 mL of distilled water to ammonium persulfate solution (0 1 g) and allow to dissolve, then add 211 µL of solution to the squeezy bottle. Then add 14 µL of TEMED to squeezy bottle and thoroughly mix.
6. Put plates horizontally onto block with indented plate uppermost. Keeping the plate horizontal, slowly add gel to plate while trying to avoid bubbles. Carefully add the comb (either 24- or 36- well) to the plate at an angle to avoid bubbles.
7. Leave plate to set for at least 1–2 h. Wash squeezy bottle and measuring cylinder with water

3.2.2. GeneScan Sequencer

1 Switch on GeneScanner and wait a few minutes for warm-up and calibration cycle to end. Press *main menu* then *set up run*, change the run time (dependant on size of primers but usually approx 3 h for primers up to approx 400 bp). Switch on floptical disk and then Mac computer, then click on *collection*.
2 Press *main menu*, *calibration* then *fluor check* Alter PMT setting to approx 600, press *change* then *main menu* Adjust the plate length inside lid for size of gel e.g., 12 cm.
3. Place bottom reservoir in machine. Remove clips from plates, clean plates again with water and ethanol, especially around the areas that will be scanned and around the top, then place in GeneScanner
4. Press *start pre-run*, *plate check*. After the first few scans, check that the scan lines are smooth and bottom line is at 800–900. If not, press *main menu*, then *abort run* and go back to *fluor check* above and alter PMT setting and repeat until at 800–900
5. Fit top reservoir and attach electrodes and clamps Make up 1X TBE buffer (150 mL 10X stock and top up to 1500 mL with water). Fill top reservoir with 700 mL and bottom with rest. Close lid and press start pre-run, pre-run gel. Run gel for at least 10 min.
6. While the gel is running, the samples can be prepared and denatured.

3.2.3. Sample Preparation

1. To prepare each PCR sample use· 0 6 µL GeneScan standard (350 bp standard suitable for products up to approx 300 bp, ABI), 3 5 µL formamide, 0.5 µL of loading buffer (blue dye), and 1.5 µL of PCR product. If there are 36 samples, make up enough for 40 in case of pipeting error, i.e., 24 µL standard, 140 µL formamide and 20 µL loading buffer. Add 4.5 µL of this mix to labeled Eppendorf tubes.
2. Add 1.5 µL of each PCR sample to the labeled Eppendorfs.
3. Denature all samples in PCR machine at 90°C for 2 min then indefinitely hold at 4°C

3.2.4. Computer Preparation

1. After *plate check*, close the collection file (red square) then *quit* in menu. Go into hard disk, then *GeneScan*, *GS analysis*. If a new sample sheet is not needed, go to 2.

If a new sample sheet is needed, go to *file*, *new*, then *sample sheet.* Select gel format (New amadite 6% gel). Change dye color to red. Type in gel name and click if 24 or 36 lanes Fill in details for each sample for each dye (normally type in 1 to 36 in dyes blue, green and type in any letter for all red lanes). Click on the *red for standard*, click on *file* then *save as* and then enter sample sheet folder and save the new sheet.

2 Close sample sheet and click on *analysis*, *pre process parameters*. Alter the scan sizes (normally 100 to 2000 but dependant on size of products) and change the sample to the one just created above or to a previous sample sheet.
3. Click on *analysis*, then *analysis parameters* to check minimum peak heights. Change the value of the dyes to 20 and change the others to 150 (lower numbers enhance weak signals but increase background).
4. Quit from *GS analysis*, *GeneScan,* and the hard disk, check that only finder is running before opening collection. Click on *edit, settings* and change collection length (usually 3 h for up to 400 bp fragments), and give the collection file a name.
5. After prerun for 10 min has been completed and after samples have denatured, open GeneScanner lid and wash out the lanes with a large pipet and buffer to remove excess gel

3.2.5. Loading the Samples

As the internal size standard allows staggered loading to be performed, DNA samples can be loaded in alternate wells and the gel electrophoresed for 10 min before the second set samples are loaded. This ensures that potential lane to lane spillage does not affect the subsequent quantitation and sizing. As the samples undergo electrophoresis, the laser scans across its field of view and fluorescence signals emitted by the loaded products are detected, collected, and stored using the Genescan software.

1 Carefully load the samples onto the scanner using flat-tipped eppendorf tips. Normally stagger load i.e , load odd numbers first, on scanner press *choose run* then press *GeneScan run*. Start the collection by clicking on green triangle Run the sample for 5–10 min, then abort run on scanner, and stop run on computer. Now load the even numbered samples and start from *choose run* again.
2 Wait for first few scans to appear then turn brightness down on computer screen to avoid screen burn-out. Wait for run to end in approximately 3 h.
3. At end of run, *stop run* (red square) and click *save data*. The computer will now calculate and save the data which takes approximately 5 min.
4. Copy the file from the hard disk to a floptical disk. Stop the Genescanner by pressing *abort run.*
5. Remove the top reservoir and throw buffer away. Remove gel plates and slowly prise the two plates apart using the thick comb. Roll the gel off the plates and dispose of the gel carefully.
6. Put gel plates, combs, and spacers into sink with fresh sachet of detergent and soak overnight

3.2.6. Gel Analysis

1. Click on *GeneScan data file* (looks like double helix). Adjust gel contrast by going to *gel, adjust gel contrast,* deselect all colors except red, and move baseline up and down and click *OK* to give much brighter red signals. When gel picture is formed, check that all lanes are tracked correctly. If not, move cursor to each lane *and lock on* (lock and key symbol). This may need to be done for each lane.
2. Click on *analysis* and choose *analyze lanes* Alter standard marker to red. Click and hold standard, choose user defined, and alter the lane number to one with a strong red signal. Fill in correct sizes for the red standards: (94, 109, 116, 172, 186, 222, 233, 238, 269, 286, 361, etc if using ABI 5000 standard) Then press *done.*
3. Sometimes all the sizes may not be visible because of the length of run selected. If so, put in all available sizes e g , 94 to 222 sizes only.
4. Go into *analysis* and select the colours (normally blue, green and red) for all the lanes used (normally 1–36). Press *analyse* and wait approximately 30 s per lane for each lane to be analysed.
5. When all the lanes have been completed, go to *results* and click *electrophoretogram* results. Select the lane you want to see and click on the colors (normally blue and green). It is possible to select up to four colors, but normally two are sufficient (one is the dye you have used and the other will be the red standard). Click on *show.*
6. Save work here as printing may cause the computer to crash and all the gel analysis data will be lost.
7. To print, click on *file* then choose *print one.* Printing takes 1–2 min per diagram, so if there are many to do, send them all individually to the printer, but do not send more than five at a time as the computer may crash.
8. When all analyses and printing has finished, *close results* and save any changes. The raw data file (double helix) can now be deleted safely.

3.2.7. Reanalysis of Data

1 To reanalyze the data using different scan numbers, e g., to get rid of primer dimers, go to *analysis, pre-process parameters,* and change start and end numbers depending on scan size in gel picture
2 Go to *analysis, preprocess collection file,* and open raw data file (helix) Wait for gel picture to form.
3 Retrack all lanes. Again, go to gel analysis above

3.2.8. Taking a Picture of the Screen

1. To take a picture of the screen, e.g., for importing into a Word Processing document, make sure that the screen shows only what you want to take a picture of Press shift, apple key and 3 at the same time. A picture will be created on the hard disk.
2. Exit GeneScan program and enter Teach-Text, or Word for Mac programs. Go to insert menu and insert the picture that has just been saved. Save the Word document in a form that can be used by a word-processor e.g., Word for Windows, WordPerfect, and delete original picture file.

3.2.9. Quitting the Scan

1. When finished, *quit analysis*, GeneScan and check that only finder is on.
2. Go to special and *empty trashcan.*
3. Eject floptical by dragging icon to trashcan. If all printing has stopped, go to special, and then *shutdown.*

4. Notes

1 If several primers are used in a multiplex reaction, each primer may have different requirements, so a compromise must be reached allowing sufficient yield from each reaction.
2. High *Taq* concentration results in amplification of nonspecific background products, while insufficient *Taq* reduces product yield.
3. In general, product yield from PCR is exponential until approx 30–40 cycles, therefore, cycles number should not exceed 40. Denaturation, annealing and extension times, and temperatures also require optimization to ensure optimal strand separation and hybridization efficiency without loss of enzyme efficiency.

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21

PCR-Based Noninvasive Prenatal Diagnosis Using Fetal Cells in Maternal Circulation

Y. M. Dennis Lo

1. Introduction

It is a long-term goal of human genetics to develop prenatal diagnostic tests that are noninvasive and which do not constitute a risk to the mother or fetus. Such tests would be highly desirable compared with current techniques that require the sampling of fetal tissues by invasive measures such as amniocentesis and chorionic villus sampling (CVS) that represent some risk to the fetus. One potentially noninvasive source of fetal cells for genetic analysis is fetal cells that have entered into maternal circulation during pregnancy *(1,2)*.

The existence of fetal cells in maternal blood has been postulated since 1893 when Schmorl observed the presence of "trophoblastic islands" in the lungs of women who died of eclampsia *(3)*. This observation suggests that fetal cells gain access into maternal circulation and subsequently embolize into maternal lungs. Since then, a large number of investigators have been trying to demonstrate these cells in maternal peripheral circulation. Techniques that have been attempted include cytogenetics *(4)*, quinacrine staining *(5)*, and fluorescence activated cell sorting *(6)*. These techniques have met with mixed success. Early attempts of using modern molecular biology to confirm the existence of fetal cells in maternal blood have been disappointing, showing either that the cells isolated using these early techniques are of maternal origin *(7)* or that current techniques are not sensitive enough to detect these rare fetal cells *(8,9)*.

The situation changed when in 1989, Lo et al. developed a nested polymerase chain reaction (PCR) method to detect fetal-derived Y chromosomal sequences from peripheral blood of women bearing male fetuses *(10)*. This

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study represents the first molecular evidence that nucleated fetal cells indeed circulate in maternal blood, and, furthermore, these cells can be used to obtain genetic information regarding the fetus. Subsequently, PCR has been shown to be sensitive enough to detect a single copy fetal gene from maternal blood *(11)*. Since then, PCR has been used to diagnose haemoglobin Lepore-Boston disease *(12)*, fetal rhesus D status *(13,14)*, and other paternally inherited DNA polymorphisms *(15)* from maternal peripheral blood. However, the use of PCR alone for noninvasive prenatal diagnosis does pose certain limitations, first, because current techniques have not yet achieved the precision required for clinical diagnosis, second, the PCR approach can only be used to detect paternally inherited mutations or polymorphisms (as the rare fetal cell is surrounded by an excess of maternal cells), and, third, the diagnosis of chromosomal aneuploidy is best diagnosed by a technique other than PCR.

These limitations have prompted investigators to develop techniques to enrich the fetal cells. Different groups have concentrated on different target fetal cells for isolation, including fetal nucleated red cells *(16,17)* and trophoblasts *(18)*. Using these methods, it is possible to diagnose chromosomal aneuploidies from maternal blood *(19,20)*. Very recently, an approach based on the culture of fetal red cell precursors has also been reported *(21)*. The discussion of these approaches is beyond the scope of this chapter and here I shall review several PCR-based systems for detecting fetal DNA sequences from maternal blood. These techniques can be combined with the enrichment processes mentioned above to produce a more accurate system.

The three systems discussed below are all based on the principle of detecting a DNA sequence that is possessed by the fetus but not by the mother. Thus, the DYS14 Y-PCR system aims to detect a Y-specific sequence that is present in a male fetus that the mother does not possess *(11,22)*. The rhesus D PCR system is based on the principle that rhesus D-positive individuals possess the rhesus D gene that is absent in rhesus D-negative individuals *(13,14,23)*. The third system is based on the existence of a polymorphic 3.1-kb region 5' to the human δ-globin gene *(24,25)*. There are 18 polymorphic sites clustered in this region, but only in two combinations (i.e., haplotypes). When used in noninvasive prenatal diagnosis, the objective is to detect a fetal haplotype that is not possessed by the mother.

2. Materials

1. A thermal cycler is necessary for the PCR. The author uses either a Biometra TRIO (Gottingen, Germany) thermoblock or a Perkin-Elmer DNA Thermal Cycler.
2. Reagents for the PCR, including the *Thermus aquaticus* (*Taq*) DNA polymerase, deoxynucleotides (dNTPs), Ampliwax for hot start PCR *(26)* and PCR buffer are obtained from a GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer, Foster City, CA).

Table 1
Primer Sequences

Primer	Sequence
Y1.5	5'CTA GAC CGC AGA GGC GCC AT3'
Y1.6	5'TAG TAC CCA CGC CTG CTC CGG3'
Y1.7	5'CAT CCA GAG CGT CCC TGG CTT3'
Y1.8	5'CTT TCC ACA GCC ACA TTT GTC3'
RD-A3	5'GGA TTT TAA GCA AAA GCA TCC AAG AA3'
RD-2	5'ACT GGA TGA CCA CCA TCA TAT3'
RD-5	5'CAA GGC CTG TTC AAA AAC AAG3'
ARMS 757R	5' TAC TGA GTT CTA AAA TCA GCT3'
ARMS 757T	5' TAC TGA GTT CTA AAA TCA TCG3'
ARMS 894R	5' ATT CAC AGT TAT ATC TAA ACT CTT ATA GAT3'
ARMS 919T	5' AGG TTG GAG GTC AGA GGT TAG AAA TCA CAG3'
ARMS 1154T	5' TAA TAG ATA GGG ACA AAT TGA AGC AGA AGC3'
COMM 1223	5' GGA AAG AAT AGC ATC TAC TCT TGT TCA GGA3'
COMM639	5' CCT TTT TGT TTC AGC TTT TCA CT GTG3'

3. Oligonucleotide primers for the PCR were obtained from Genosys (Cambridge, UK). Three PCR systems are described as examples, namely the amplification of the DYS14 locus (a Y-specific sequence) *(27)*, the amplification of the rhesus D gene *(28)*, and the amplification of a polymorphic region 5' to the human δ-globin gene (referred in this chapter as the RT region) *(15,24,25)*. Primer sequences are listed in **Table 1** and primer locations are shown in **Figs. 1–3**. The primer combinations Y1.5/Y1.6 (product size 239 bp) and Y1.7/Y1.8 (product size 198 bp) are nested with respect to one another, being the external and internal pair, respectively (**Fig. 1**). The primer combinations RD-A3/RD2 (product size 291 bp) and RD-5/RD-2 (product size 262 bp) are both specific for the RhD gene and are heminested with respect to each other, with the latter primer combination being the internal set (**Fig. 2**). The allele-specific primers for typing the RT region are designed using the principles of the Amplification Refractory Mutation System (ARMS) *(29)* (*see* Chapter 7). The RT region can be one of two haplotypes, the R or the T type, and ARMS primers designed to prime specifically each type is suffixed by the target type (*see* **Note 6** and **Fig. 4**). The RT system can only be used to detect fetal cells in maternal blood in informative families. A family is informative if the fetus possesses an allele that the mother does not possess. For this to occur, the mother must be homozygous for either the R haplotype (i.e., RR) or the T haplotype (i.e., TT). In these cases, the family is informative if the fetus is heterozygous for the region (i.e., RT). It is obviously biologically impossible for the mother to be homozygous for one haplotype and the fetus to be homozygous for the other haplotype.

DYS14 locus

Fig 1 Schematic diagram showing the location of the primers for the DYS14 Y-specific amplification system.

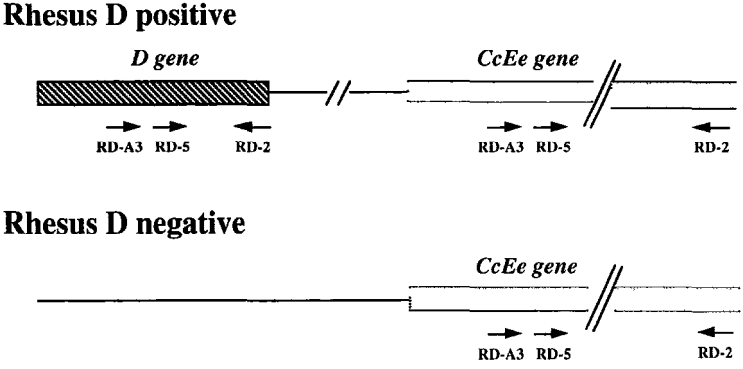

Fig. 2. Schematic representation illustrating the genetic difference between rhesus D-positive and rhesus D-negative individuals. The former possess the rhesus D gene that is absent in the latter Primer locations are indicated in the diagram.

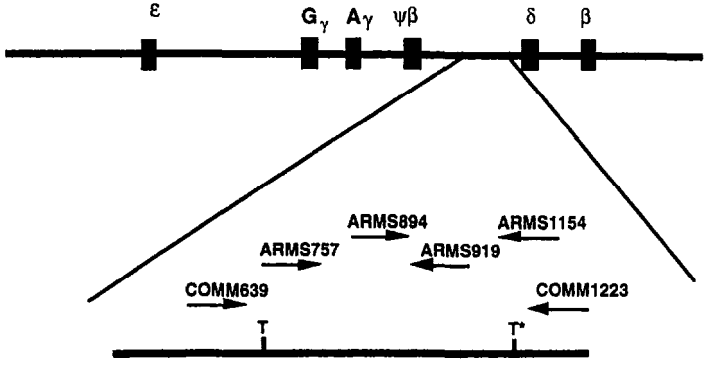

Fig. 3. Schematic representation of the polymorphic region 5' to the δ-globin gene. The relative locations of the primers are illustrated. The prefix ARMS indicates an ARMS primer whereas the prefix COMM represents a non-ARMS primer. The number suffix of the primer name indicates the position of the last base of the primer. Numbering of the sequence is as described by Maeda et al. *(24)*. Region marked by T denote recognition sites for the restriction enzyme *Taq*I. The *Taq*I site marked by * is polymorphic and is only present on T chromosomes.

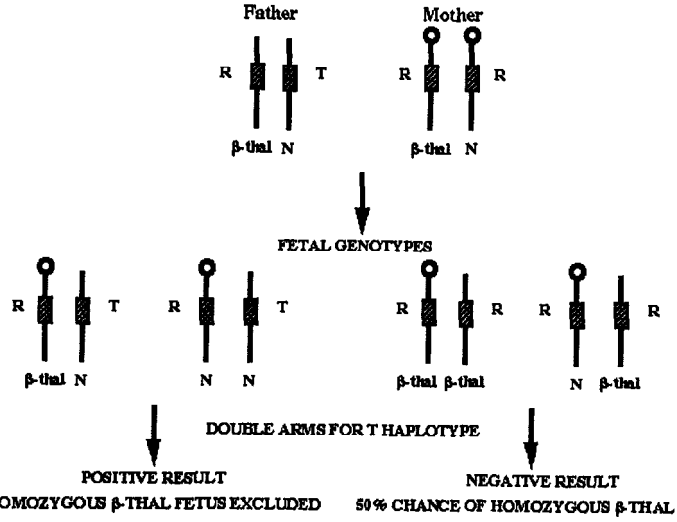

Fig 4 Application of double ARMS to noninvasive prenatal diagnosis of β-thalassemia β-thal, mutant β-globin gene causing β-thalassemia; N, normal β-globin gene; R, R haplotype; T, T haplotype. The figure illustrates one example of an informative family

Typing of individuals for the RT region can be easily carried out by amplification of a segment of the region using primer COMM639 and COMM1223, followed by cutting with the *Taq*I restriction enzyme *(30)*. The T haplotype possesses a *Taq*I site at position 1152 (numbering according to Maeda et al. *[24]*) that is absent in the R haplotype.

For the detection of a RT fetus from the peripheral blood of a TT mother, the following primer combinations are used: ARMS757R and COMM1223 for 30 cycles followed by a nested round of amplification using ARMS894R and COMM1223 for another 30 cycles. The thermal profile for the amplification reactions are listed in **Table 2**.

For the detection of a RT fetus from peripheral blood of a RR mother, the following primer combinations are used: ARMS757T and ARMS1154T for 30 cycles followed by a nested round of amplification using ARMS757T and ARMS919T for another 30 cycles. The thermal profile for the amplification reactions are listed in **Table 2**.

3. Method

1. Collect peripheral blood samples from pregnant women either into EDTA or lithium heparin tubes (*see* **Notes 3** and **4**). Perform DNA extraction by the standard proteinase K, phenol-chloroform method *(31)*. Carry all extraction and set up procedures in a laminar flow cabinet (*see* **Note 1**).

Table 2
Thermal Profile and Mg^{2+} Concentration for the Primer Combinations

Primer combination	Thermal profile (denaturation, annealing, extension)	Optimal Mg^{2+} concentration, m*M*
Y1.5/Y1.6	94°C 1 min, 55°C 1 min, 72°C 1 min	1.5
Y1.7/Y1 8	94°C 1 min, 55°C 1 min, 72°C 1 min	1.5
RD-A3/RD-2	94°C 1 min, 55°C 1 min, 72°C 1 min	1.5
RD-5/RD-2	94°C 1 min, 55°C 1 min, 72°C 1 min	1.5
ARMS757R/COMM1223	94°C 1 min, 55°C 1 min, 72°C 1 min	4
ARMS894R/COMM1223	94°C 1 min 57°C 1 min, 72°C 1 min	4
ARMS757T/ARMS1154T	94°C 1 min, 57°C 1 min, 72°C 1 min	2
ARMS757T/ARMS919T	94°C 1 min 57°C 1 min, 72°C 1 min	1 5
COMM639/COMM1223	94°C 1 min, 57°C 1 min, 72°C 1 min	1.5

2. Set up PCR in 100 µL reaction volumes with 2.5 U of *Taq* DNA polymerase in each tube (*see* **Note 1**). The optimal $MgCl_2$ concentration for the individual amplification systems are listed in **Table 2**. Use one to five µg of genomic DNA extracted from pregnant women per reaction (*see* **Note 5**). For Y-specific amplification, first round PCR consists of 40 cycles using the primer pair Y1.5 and Y1.6. Transfer 2 µL of the first round PCR product into fresh reagents and reamplify for 25 cycles using the same thermal profile with the internal primer pair Y1.7 and Y1.8. For RhD amplification from maternal peripheral blood, 50 cycles of Ampliwax-mediated hot start PCR are carried out using RD-A3 and RD-2. Reamplify 2 µL of this first round reaction using RD-5 and RD-2 for a further 10 cycles using the same cycling parameters. To detect a R allele from a RT fetus carried by a TT mother, carry out 30 cycles of first round PCR using ARMS757R and COMM1223. Reamplify 2 µL of this first round reaction using ARMS 894R and COMM1223 for a further 30 cycles. To detect a T allele from a RT fetus carried by a RR mother, carry out 30 cycles of first round PCR using ARMS757T and ARMS1154T. Reamplify 2 µL of this first round reaction using ARMS 757T and ARMS919T for a further 30 cycles.
3. Electrophorese 10 µL of PCR products using a 1.5% ethidium bromide stained agarose gel. Visualize PCR products under UV transillumination.

4. Notes

1. The sensitivity of PCR is both its strength and its weakness because it is very prone to contamination *(32)*. For the detection of fetal cells in maternal blood, a sensitivity of at least one target cell per 10^5 background cells is necessary At this and higher sensitivity levels, it is necessary to be obsessive regarding anti-contamination procedures (*see* Chapter 2 on PCR contamination) The basic precautions include the use of aliquoted reagents, the physical separation of the pre- and post-PCR areas and equipment, the use and frequent change of gloves, and the use of aerosol-free pipet tips. Negative controls should be included in every experiment. In many situations, multiple negative controls are preferable to a single one. Negative controls should include water blanks to test for reagent contamination as well as appropriate genomic DNA controls, e.g., female cord blood DNA for Y-PCR using the DYS14 system, rhesus negative male DNA for the RhD system, and male RR or TT homozygote for ARMS PCR for the RT region.
2. The detection of fetal DNA sequences from maternal blood has not yet achieved the precision necessary for clinical diagnosis. Different investigators have reported different accuracy rates *(22,33–37)*. The exact reason for this difference in diagnostic accuracy is unclear at present, but may be related to the differences in amplification targets and amplification protocols employed by different groups It is likely that in the future, fetal cell enrichment techniques will improve the accuracy of this noninvasive approach of prenatal diagnosis.
3. There is a theoretical risk that fetal cells may persist from one pregnancy into subsequent pregnancies. However, current evidence suggests that for methods with detection sensitivity in the order of 1 fetal cell in 300,000 maternal cells (e.g., the PCR-based methods described in this chapter), fetal cells become undetectable by 8 wk post-partum *(22,37)* and thus are unlikely to affect the diagnostic accuracy in tests carried out in subsequent pregnancies. There are, however, data in the literature that suggest that under very special conditions, such as the enrichment of fetal hemopoietic stem cells from maternal blood, fetal cell persistence may be detectable in some cases *(38)*.
4. Fetal cells have been found in maternal circulation during the first trimester, as early as 4 wk and 5 d *(37)*. Some investigators have reported a trend of increased fetal cell numbers as the pregnancy progresses *(34)*.
5. The amount of DNA used for PCR is important, as this quantity is a reflection of the number of maternal cells (and hence the volume of maternal blood) among which one is searching for the rare fetal cells. Thus, if the amount of DNA is reduced, then one is effectively reducing the chance of finding the rare fetal cells. As 1 µg of genomic DNA corresponds to approx 150,000 diploid cells, at single molecule sensitivity, this corresponds to a sensitivity of 1 in 150,000. This consideration, however, has to be balanced against the fact that increasing the amount of DNA will lead to the increase in the number of non-specific amplification bands and to the increase in PCR inhibition due to inhibitors existing in genomic DNA. As a guide, maternal DNA from 1–5 µg provides satisfactory results.

6 The RT polymorphic region is closely linked to the β-globin gene and can be used in the prenatal diagnosis of β-thalassemia by linkage analysis. One example that illustrates the principle underlying this type of analysis is shown in **Fig. 4.** This example shows the most informative scenario in which the definitive diagnosis of an unaffected child (i.e., either homozygous normal or a carrier of β-thalassemia) can be positively diagnosed from maternal peripheral blood. If the system can be shown to be reliable then no further prenatal diagnostic testing would be needed

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22

Noninvasive Prenatal Diagnosis Using a Single Fetal Nucleated Erythrocyte Isolated by Micromanipulation from Maternal Blood

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1. Introduction

Prenatal diagnosis of hereditary diseases is usually performed by collecting fetal samples using amniocentesis, chorionic villus sampling (CVS), or cordocentesis. However, these procedures are associated with some degree of risk. For example, abortion owing to hemorrhage or infection occurs in 0.2–0.4% of patients undergoing amniocentesis. Furthermore, CVS reportedly presents the potential risk of fetal limb malformation in 0.01–0.03% of cases *(1)*. Each method has the risk of misdiagnosis because of contamination by maternal cells. Thus, the development of a suitable noninvasive method is important (**Table 1**).

Various fetal cells, such as trophoblasts *(2–3)*, erythrocytes *(4)*, and leukocytes *(5)* are known to exist in the maternal circulation from an early stage of pregnancy, and thus can provide a source of fetal genes for prenatal diagnosis. However, fetal trophoblasts and leukocytes appear at minute concentrations in maternal circulation. Furthermore, fetal lymphocytes are difficult to distinguish from maternal lymphocytes and continue to exist for several years after delivery *(6)*. As a result, fetal trophoblasts and leukocytes do not appear to provide a practical means of prenatal diagnosis. On the other hand, fetal nucleated erythrocytes (NRBCs) may be suitable for prenatal diagnosis, because they are not commonly found in the peripheral blood of normal adults and are the most abundant fetal cells found in maternal circulation. Recently, many investigators have attempted to isolate fetal cells from maternal blood by various cell separation techniques, including fluorescence-activated cell sorting *(3,7–8)*,

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Table 1
Summary of Prenatal Diagnostic Methods

Diagnostic methods	Weeks of gestation	Benefits	Defects	Fetal loss rate, %
Amniocentesis	15–18	Relatively simple	Necessary to culture Contamination by maternal cells	0 2–0.4
Chorionic villus sampling	9–12	Early and direct diagnosis possible	Limb malformations (0 01–0.03%) Contamination of maternal cells Placental mosaicism	0 5–1 0
Cordocentesis	18–	Early and direct diagnosis possible	Bleeding Fetal bradycardia Contamination of maternal cells	0.5–2.0
Maternal peripheral blood	6–	Noninvasive	—	0

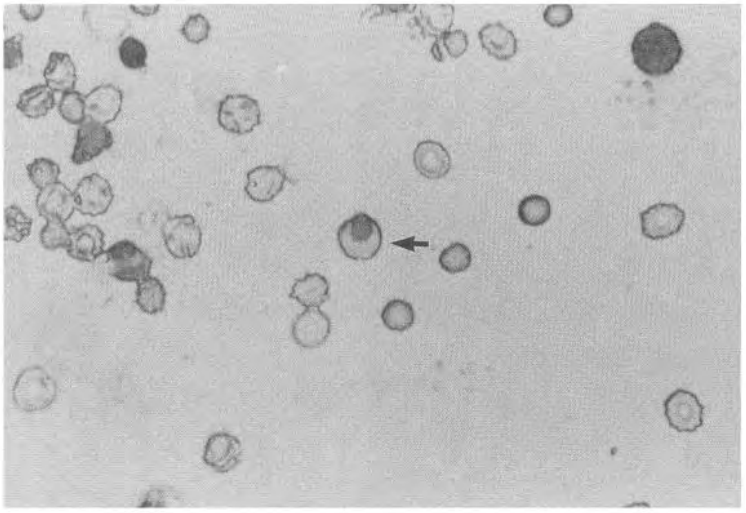

Fig. 1. Nucleated erythrocyte obtained from maternal peripheral blood on a glass slide.

and magnetic-activated cell sorting ***(9)***. However, it had not been possible to retrieve fetal cells selectively and individually at the single-cell level until the development of a technique for retrieving NRBCs from maternal blood using a discontinuous density gradient method with Percoll and a micromanipulator under microscopic observation, as reported by Takabayashi et al. in 1995 ***(10)***. NRBCs can also be collected from peripheral blood samples of pregnant women at 8–11 wk of gestation. **Figure 1** shows a single NRBC in maternal blood at 9 wk of gestation.

Polymerase chain reaction (PCR) is sensitive enough to allow analysis of DNA in a single cell. Furthermore, primer extension preamplification (PEP) developed by Zhang et al. ***(11)*** provides an in vitro method for amplifying a large fraction of DNA sequences present in a single haploid cell by repeated primer extension using a mixture of 15-base random oligonucleotides. It was also reported that at least 78% of the genomic sequences in a single human haploid cell can be copied by this method, and then multiple small aliquots can be removed for individual analysis using a nested or hemi-nested PCR of numerous genetic loci.

We applied these methods to the prenatal DNA diagnosis of Duchenne muscular dystrophy (DMD) ***(12)*** as well as the determination of fetal RhD blood type ***(13)*** using a single NRBC obtained from maternal peripheral blood. After amplification of single NRBCs using the PEP method, 60 μL of the reactants are divided into aliquots to allow multiple DNA analysis by PCR.

The ZFX/ZFY gene, located in the sex chromosome *(14)*, is then amplified using a nested PCR of the 2-μL PEP aliquot in order to confirm whether the whole genome is amplified by PEP and to establish that the NRBC indeed originated from the fetus. If the NRBC is male, the cell can be genetically confirmed to be of fetal origin. The deletion of DMD exons, and RhD genes can be further analyzed from the same aliquots of PEP reactants. Thus, this method allows prenatal DNA diagnosis using single NRBCs from maternal circulation.

2. Materials

2.1. Isolation of Fetal NRBCs from Maternal Blood

2.1.1. Sample Collection

1. Subjects consisted of pregnant women at 8–20 wk gestation (*see* **Note 1**)
2. Collect 7 mL of maternal peripheral blood into ethylene diaminetetra-acetic acid 2Na (EDTA-2Na) coated tubes.

2.1.2. Collection of Fetal NRBC Rich Layer from Maternal Blood (see **Note 2**)

1. 1.075 and 1.085 g/mL Percoll gradients: Prepare Percoll gradient solutions at the densities of 1.075 and 1.085 g/mL by mixing either 53.1 or 60 8 mL of 1.129-g/mL Percoll solution (Pharmacia Biotech, AB, Uppsala, Sweden) with 10 mL of 1.5 *M* NaCl and 36.9 or 29.2 mL of distilled water (DW), respectively. Store at 4°C for up to 3 mo
2. Phosphate-buffered saline (PBS), pH 7.4: Dissolve 8 g NaCl, 2.9 g Na_2HPO_4 $12H_2O$, 0.2 g KCl, 0.2 g KH_2PO_4 in DW, making a final volume of 1 L. Store at room temperature (RT) after autoclaving.
3. Methanol (highest grade).
4. Giemsa stain solution: Dilute 100 μL of Giemsa stain solution with 10 mL of DW. Dilute just prior to use.

2.1.3. Isolation of Single NRBCs Using a Micromanipulator

1. Micromanipulation setup: Microscope (Olympus IX-70, Tokyo, Japan) with micromanipulator (Narishige, Tokyo, Japan).
2. TE buffer (pH 8.0): Mix 1 mL of 1 *M* Tris-HCl, pH 8 0 with 0.2 mL of 0.5 *M* EDTA (pH 8.0) and make up to 100 mL with DW. Sterilize by autoclaving and store at RT.
3. Trypsin (0.25%): Diluted with TE buffer. Stable at –20°C for up to 1 yr.

2.2. Amplification of Single NRBCs (see Note 4)

2.2.1. Whole Genome Amplification

1. TE buffer (pH 8.0): Mix 1 mL of 1 *M* Tris-HCl, pH 8.0 with 0.2 mL of 0 5 *M* EDTA (pH 8.0) and make up to 100 mL with DW. Sterilize by autoclaving and store at RT

2. Alkaline lysis solution: 200 m*M* KOH, 50 n*M* dithiothreitol. Stable at –20°C for up to 1 yr.
3. Neutralization solution: 900 m*M* Tris-HCl, pH 8.3, 300 m*M* KCl, and 200 m*M* HCl. Stable at –20°C for up to 1 yr.
4. 400 μ*M* Random primer: (5'-NNNNNNNNNNNNNN-3', Operon Technologies, Alameda, CA) Dissolve random primer in TE buffer to final concentration of 400 μ*M*. Stable at –20°C for up to 1 yr.
5. *Taq* DNA polymerase (Takara, Tokyo, Japan).
6. 10X K^+ free-PCR buffer: 25 m*M* $MgCl_2$, 1 mg/mL gelatin, and 100 m*M* Tris-HCl, pH 8.3. Stable at –20°C for up to 1 yr.
7. dNTPs solution: 2.5 m*M* of each dNTP. Stable at –20°C for up to 1 yr.
8 Thermocycler (Astec, Tokyo, Japan).

2.2.2. (Hemi-) Nested PCR

1. 10X PCR buffer: 15 m*M* $MgCl_2$, 500 m*M* KCl, and 100 m*M* Tris-HCl (pH 8.3). Stable at –20°C for up to 1 yr.
2. Primers for sex determination.
   a. Outer primers.
      (Sense): 5'-ACCA/GCTGTACTGACTGTGATTACAC-3'
      (Antisense) · 5'-GCACC/TTCTTTGGTATCC/TGAGAAAGT-3'
   b Inner primers:
      (Sense) 5'-AC/TAACCACCTGGAGAGCCACAAGCT-3'
      (Antisense) : 5'-TGCAGACCTATATTCA/GCAGTACTGGCA-3'
3 *Hae*III (Toyobo, Japan)
4. Primers for diagnosis of dystrophin gene and RhD blood type (**Table 2**).

2.2.3. Electrophoresis

1. 10 mg/mL Ethidium bromide (Sigma, St. Louis, MO).
2. Gel-loading buffer (6X dye): 25 mg of 0.25% bromophenol blue, 25 mg of 0.25% xylene cyanol FF, and 3 mL of 30% glycerol are adjusted to 10 mL with DW.
3. 0.5X TBE buffer: 44.5 m*M* Trizma base, 44.5 m*M* boric acid, and 1 m*M* EDTA-2Na in final concentration.
4. 2.5% agarose gel: Dissolve 3.75 g of ultra-pure DNA grade agarose (FMC, Rockland, ME) in 150 mL of 0.5X TBE. Heat in a microwave oven. After cooling down to 65°C, add ethidium bromide to make a final concentration of 0.5 μg/mL. Pour agarose gel into a gel maker set (Advance, Tokyo, Japan) and cool to RT.

3. Methods

3.1. Isolation of Fetal NRBCs from Maternal Blood

3.1.1. Blood Sampling

1. Collect blood samples (7 mL) in EDTA-2Na-coated tubes from peripheral veins of pregnant women

Table 2
Primer Sequences

Exon	Primer name[a]	Sequences (5'–3')
Primers for DMD		
4	1F	TTGTCGGTCTCCTGCTGGTCAGTG
	2R	CTGTGTCACAGCATCCAGACCTTGT
	3R	CAAAGCCCTCACTCAAACATGAAGC
8	1F	GTCCTTTACACACTTTACCTGTTGAG
	2F	TCATGGACAATTCACTGTTCATTAA
	3R	GGCCTCATTCTCATGTTCTAATTAG
12	1F	GATAGTGGGCTTTACTTACATCCTTC
	2R	TATGTTGTTGTACTTGGCGTTTTAG
	3R	GAAAGCACGCAACATAAGATACACCT
45	1F	AAACATGGAACATCCTTGTGGGGAC
	2F	GCTCTTGAAAAGGTTTCCAACTAAT
	3R	CATTCCTATTAGATCTGTCGCCCTAC
48	1F	TTGAATACATTGGTTAAATCCCAACATG
	2R	AATGAGAAAATTCAGTGATATTGCC
	3R	CCTGAATAAAGTCTTCCTTACCACAC
51	1F	GAAATTGGCTCTTTAGCTTGTGTTTC
	2R	GGAGAGTAAAGTGATTGGTGGAAAATC
	3R	TACTTGTCCAGGCATGAGAATGAGAG

Round	Primer name[a]	Sequences (5'–3')
Primers for RhD (exon 7) and RhCE (exon 7)		
First	1F	CCACAGG(G/T)GTGTTGTAACCGAGT
First	2R	AGTGACCCACATGCCATTGC
Second (RhD)	3F	CCACAGCTCCATCATGGGCTACAA
Second (RhD)	4R	TTGCCGGCTCCGACGGTATC
Second (RhCE)	5F	ATTCACCACATCTCCGTCATGCA
Second (RhCE)	6R	CACATGCCATTGCCGTTCCA

[a]1, 3, outer and inner primer, 2, inner primer; F, sense primer, R, antisense primer.

*3.1.2. Collection of Fetal NRBC Rich Layer from Maternal Blood (see **Note 6**)*

1. Dilute aliquots of maternal blood collected in EDTA with 10 mL of PBS.
2. Gently layer the diluted blood onto discontinuous-density Percoll gradients (1.075 and 1.085 g/mL)

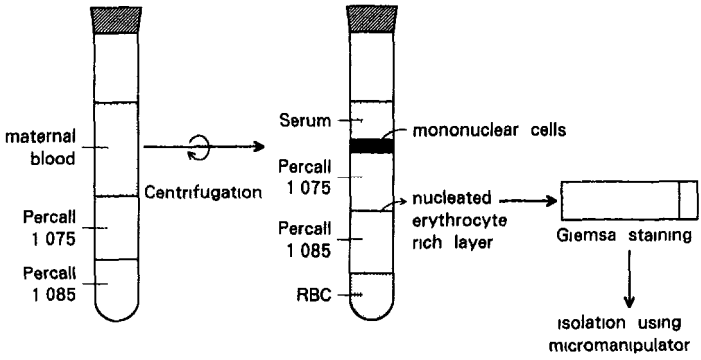

Fig. 2. Collection of fetal nucleated erythrocyte rich layer from maternal peripheral blood.

3. Centrifuge at 400*g* for 30 min.
4. Collect the interface layer of the 1.075 and 1.085 g/mL Percoll gradients.
5. Wash with 10 mL of PBS three times.
6. Centrifuge at 400*g* for 10 min
7. Remove supernatant.
8. Add a small amount of PBS to the precipitate and mix
9 Apply a thin smear to a microscope slide.
10. Fix with 100% of methanol for 10 min.
11. Stain with Giemsa for 5 min (**Fig. 2**).

3.1.3. Isolation of Single NRBCs Using a Micromanipulator

1. Using a microscope, search for the NRBCs on the slide.
2. Put one drop of 0.25% trypsin on the cell.
3. Leave at room temperature for 5 min.
4. Retrieve a single NRBC into a glass capillary pipet using a micromanipulator under microscopic observation.

3.2. Amplification of Single NRBCs

3.2.1. Whole Genome Amplification

1. Collect a single NRBC into a microtube containing 5 µL of alkaline lysis solution.
2. Incubate at 65°C for 10 min.
3. Neutralize by adding 5 µL of neutralization solution.
4. Amplify DNA from the cell by primer extension preamplification (PEP) by adding 5 µL of a 400 µ*M* solution of random primers, 6 µL of 10X K^+ free PCR buffer, 3 µL of a mixture of the dNTPs, and 1 µL of *Taq* polymerase to the sample. Add DW to make up to a final volume of 60 µL.
5 Carry out 50 primer-extension cycles in a thermocycler. Each cycle consists of a 1-min denaturation step at 92°C, a 2-min annealing step at 37°C, programmed ramping steps of 10 s per degree to 55°C, and then a 4-min incubation at 55°C for polymerase extension

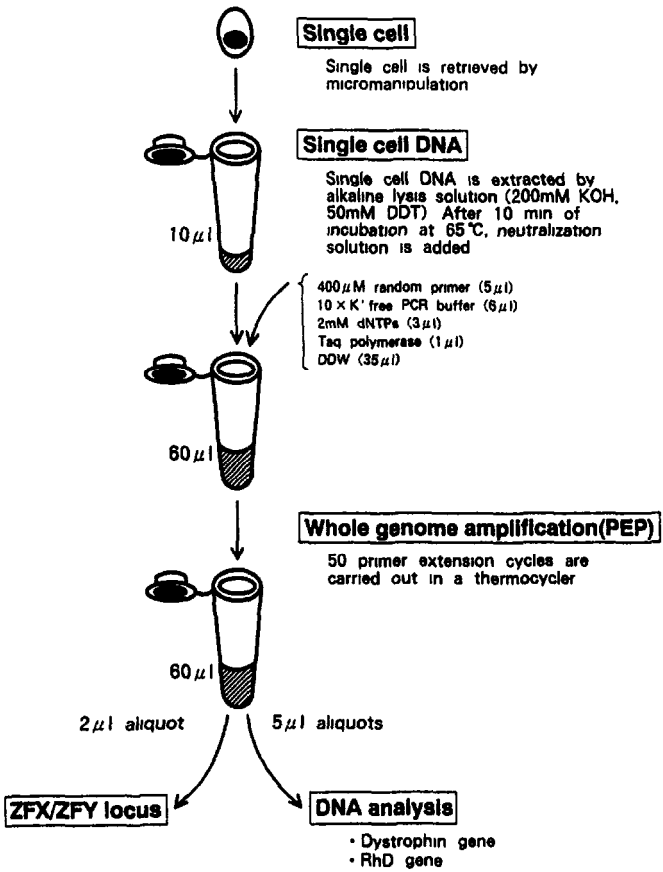

Fig. 3. The procedure of amplification of single cell using a primer extension preamplification (PEP).

6. Divide each sample into aliquots, upon which multiple DNA analysis is performed using PCR (**Fig. 3**).

3.2.2 (Hemi-) Nested PCR

3.2.2.1. Sex Determination at ZFX/ZFY Loci (*see* **Note 7**)

1. Add 2.5 μL of 10X PCR buffer, 2 μL of dNTPs, 1 μL of 10 μ*M* outer primers, and 0.2 μL of *Taq* polymerase to a 2-μL PEP aliquot. Make the final volume up to 25 μL with DW.
2 Cover samples with 30 μL of mineral oil.
3. Heat at 97°C for 2 min to ensure DNA denaturation.
4. Carry out 20 cycles of denaturation at 94°C for 45 s, annealing at 49°C for 1 min, and extension at 72°C for 1 min

5. For nested amplification, tranfer 1 µL of the amplified product to a new reaction tube containing fresh mixture with the exception that 1 µL of the 10 µ*M* inner primers is used instead of the outer primers.
6. Denature the fresh samples at 97°C for 1 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min.
7. Digest 8-µL aliquots of nested PCR products with *Hae*III in a total volume of 10 µL at 37°C for 30 min.

3.2.2.2. Analysis of Dystrophin Gene Deletions (*see* **Note 8**)

1. Add 5 µL of 10X PCR buffer, 4 µL of dNTPs, 2 µL of 10 µ*M* outer primers, and 2.5 U of *Taq* polymerase to 5 µL of the PEP aliquot. Make up to a final volume of 50 µL with DW.
2. Cover samples with 30 µL of mineral oil.
3. Denature at 94°C for 2 min, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min, and extension at 65°C for 1 min.
4. For second-stage amplification, transfer 2 µL of the amplified product to a new reaction tube containing the same mixure as described in **step 1** with the exception that 1 µL of the 10 µ*M* inner primers is used instead of the outer primers.
5. Denature at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 65°C for 1 min.

3.2.2.3. Analysis of RhD Blood Type

1. Add 5 µL of 10X PCR buffer, 4 µL of dNTPs, 2 µL of 10 µ*M* outer primers, and 2.5 U of *Taq* polymerase to 5 µL of the PEP aliquot Make up to a final volume of 50 µL with DW.
2. Cover samples with 30 µL of mineral oil.
3 Denature at 97°C for 3 min, followed by 20 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min.
4. For second-stage amplification, transfer 2 µL of the amplified product to a new reaction tube containing the same mixture as described in **step 1** with the exception that 2 µL of the 10 µ*M* inner primers for RhD and RhCE is used instead of the outer primers.
5 Denature at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 62°C for 1 min, and 72°C for 1 min.

3.2.3. Electrophoresis

1. Electrophorese the digestion products of the nested or hemi-nested PCR products on a 2.5% agarose gel in 0.5X TBE with ethidium bromide (0.5 µg/mL gel).
2. Visualize using an ultraviolet transilluminator. In sex determination, when two bands of 300 and 44 base pairs (bp) are present, a cell is considered to be genotypically female. In the case of a male, two additional bands of 216 and 84 bp are also present

4. Notes

1. We have detected fetal NRBCs from maternal blood at 6 to 40 weeks of gestation. In the present study, the number of NRBCs detected from 7 mL

of maternal blood ranged from 1–26 with an average of approx 3.0/7 mL at 8–20 wk of gestation.

2. When maternal blood is collected, the isolation of fetal NRBCs (fixation on the slide glass) should be performed within 24 h of collection.
3. After fixation, the glass slides should be stored at –20°C Isolation using a micromanipulator and amplification of the cell should be performed within a week of fixation.
4. Contamination of samples with an exogenous target DNA template can be a potential problem in PCR Therefore, appropriate measures must be taken to avoid this problem. Whenever possible, analysis should include several cells from each subject, as well as a negative control.
5. Despite the advent of PEP, which can consistently amplify a wide array of DNA sequences, it is not possible to amplify the target sequences every time because of significant locus-dependent variation in amplification efficiency. Therefore, it is first necessary to amplify the ZFX/ZFY sequences, which have a large number of repeats in single-cell DNA, and confirm whether the single cell DNA amplification is sufficient.
6. NRBCs can be found in more than 80% of maternal blood samples taken at 8–20 wk of gestation. Furthermore, sex can be determined accurately in approx 70% of single NRBCs retrieved using a micromanipulator. Genetic analysis of DMD and RhD blood type can be accomplished using aliquots of PEP reactants left over from those samples in which sex could be determined ***(12,13)***.
7. Fetal cell detection techniques described in previous reports have been limited to use in pregnancies with a male fetus. In approx 50% of pregnancies with a female fetus, the cell samples cannot be confirmed to be of fetal origin. Therefore, it is necessary to develop a new method, such as HLA typing of fetal cells, that can distinguish between maternal and fetal NRBCs.
8. Although we previously reported successful prenatal diagnosis of DMD using this procedure, prenatal DNA diagnosis using maternal blood would be most useful in situations in which the mother is a known to be a carrier of DMD due to exon deletion.

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23

Diagnosis of Chromosomal Aneuploidies Using Quantitative Fluorescent PCR

Barbara Pertl and Matteo Adinolfi

1. Introduction

1.1. Background

The incidence of chromosomal abnormalities in liveborn infants has been established to be about 1 in 170 newborns *(1)*. The most frequent chromosomal anomalies are aneuploidies involving chromosomes 21, 18, 13, and both sex chromosomes. Prenatal diagnosis of chromosomal disorders is performed by conventional cytogenetic analysis of fetal cells collected by amniocentesis, chorionic villus sampling, or fetal blood sampling. Cytogenetic techniques allow detection of chromosome aneuploidies with great accuracy. The major disadvantage of these procedures is that fetal cells must be cultured for up to two weeks before analysis. This interval of time places a significant emotional and/or clinical burden on the parents and the physician. Moreover, if the fetus is abnormal and there is a potential need for therapeutic measures, a rapid answer is of great importance. Attempts to perform rapid prenatal diagnostic tests by fluorescent *in situ* hybridization (FISH) on interphase nuclei of amniocytes are still hampered by technical difficulties *(2)*. Consequently, this approach has not yet entered into the realm of routine diagnostic procedures.

Recently, quantitative PCR amplification methods have been devised for the rapid analysis of major chromosomal disorders *(3–5)*. Highly polymorphic small tandem repeats (STRs) markers and quantitative fluorescent PCR (QF-PCR) have also been successfully applied for the detection of trisomies 21, 18, 13, and X chromosome abnormalities *(6–12)*. The QF-PCR products allow one to distinguish between normal and trisomic samples. The major advantages of this technique are its efficiency and the possibility to obtain results within 24 h.

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1.2. The Principle

The principle of QF- PCR technique is based on the presence of highly polymorphic STR markers specific for each chromosome. STRs are hypervariable regions of the genome with repeat elements of two to seven bases in length *(13)*. The polymorphic character of an STR locus results from variations in the number of tandemly repeated units from one allele to another *(14)*. Thus, amplification of polymorphic STRs results in PCR products of variable lengths in most samples. Therefore, a large proportion of individuals will be heterozygous for each of these markers. The number of alleles present in a sample can be determined by using fluorescent labeled primers for PCR amplification of STR markers specific for each chromosome followed by the quantitative analysis of the PCR products with an automated DNA sequencer. In samples from normal individuals, the PCR products will show two peaks of similar fluorescent intensities and a ratio of 1:1 corresponding to the presence of two alleles at each specific STR locus. Only a few normal samples will be homozygous for an STR marker, depending on the extent of its polymorphism. Trisomic samples will produce either three STR peaks with ratios of fluorescent intensity of 1:1:1, or two peaks with ratios of 2:1. The "triallelic" pattern with three peaks reveals the presence of three different STR alleles, whereas, the "diallelic" pattern with two peaks corresponds to the detection of two identical allele products and that of a third allele. Using several markers, specific for each potentially aneuploid chromosome, the likelihood that none of them will be informative is low. **Figure 1A,B** shows the results of testing a normal sample using markers specific for chromsomes 21, 18, 13, and both sex chromosomes. The results of testing a sample with chromosome 21 trisomy using the same set of markers are displayed in **Fig. 2A,B**.

1.3. Detection of the Common Aneuploidies by Fluorescent Quantitative PCR

This approach has been now evaluated in four studies *(8,9,11,12)*. In the first of our investigations *(9)*, trisomy 21 was diagnosed using a highly polymorphic STR marker specific for chromosome 21, D21S11. A nonpolymorphic marker was coamplified to allow the detection of normal homozygous patterns and the rare trisomic cases with three identical chromosome 21 alleles. The QF-PCR analysis of 134 samples from normal subjects and from DNA extracted from amniotic fluid, fetal blood, or fetal tissues provided clear evidence for the detection of trisomy 21. Only two samples produced abnormal STR amplification patterns; one normal sample produced three STR peaks with ratios of 1:1:2, and one trisomic sample produced 2 STR peaks with a ratio of 4:1. These pattern are so unusual that they could not lead to confusion and wrong diagnoses.

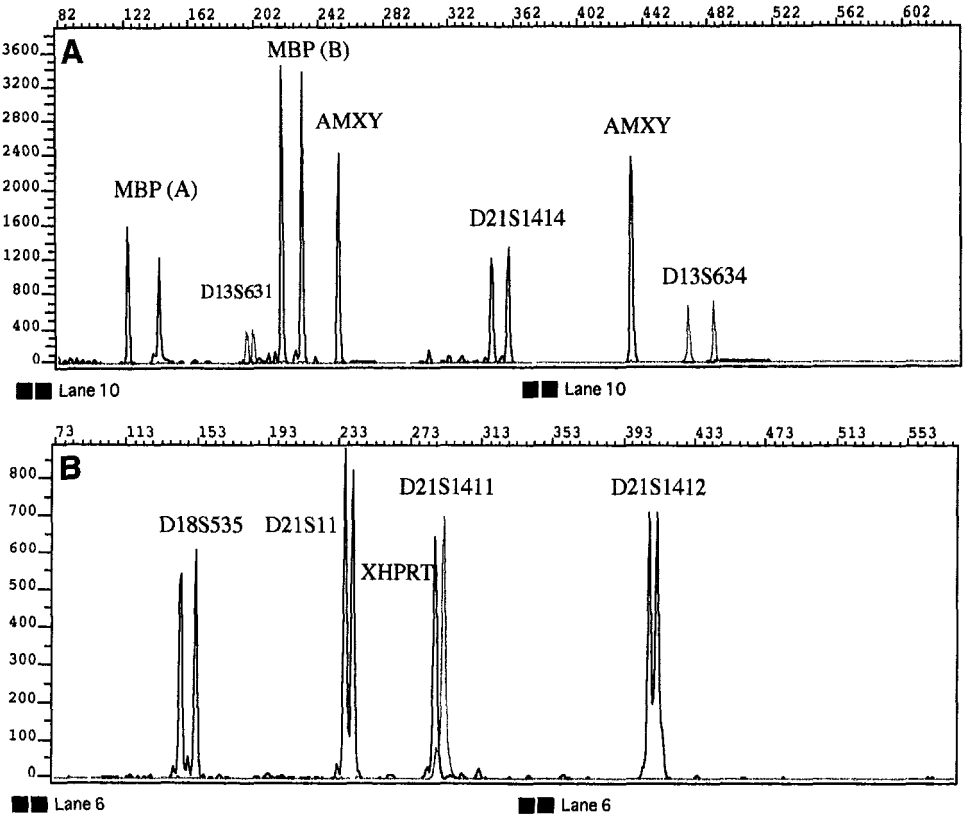

Fig. 1. **(A)** Electrophoretogram of the amplification products from a normal sample tested with STR markers specific for chromosome 21 (D21S1414), chromosome 18 (MBP Locus A and B), chromosome 13 (D13S631 and D13S634), and sex chromosomes (AMXY) (Set 1). The *x*-axis displays the computed length of the PCR products in base pairs as determined automatically by using an internal lane standard; the *y*-axis displays fluorescent intensities in arbitrary units. This control DNA is heterozygous for all markers used, the two alleles of each marker are in a 1:1 dosage ratio. Amplification of the marker AMXY results in a single peak at 433 bp corresponding to the X chromosome. **(B)** Electrophoretogram from the same normal control sample as shown in (A), using STR markers specific for chromosome 21 (D21S11, D21S1411, D21S1412), chromosome 18 (D18S535), and chromosome X (XHPRT) (Set 2). This normal DNA is homozygous at the D21S1412 locus. Amplification of all the other markers used in this set results in a normal heterozygous pattern.

The second study extended this method for the simultaneous detection of trisomies 21, 18, and sexing ***(11)***. Different samples were investigated by PCR ampli-

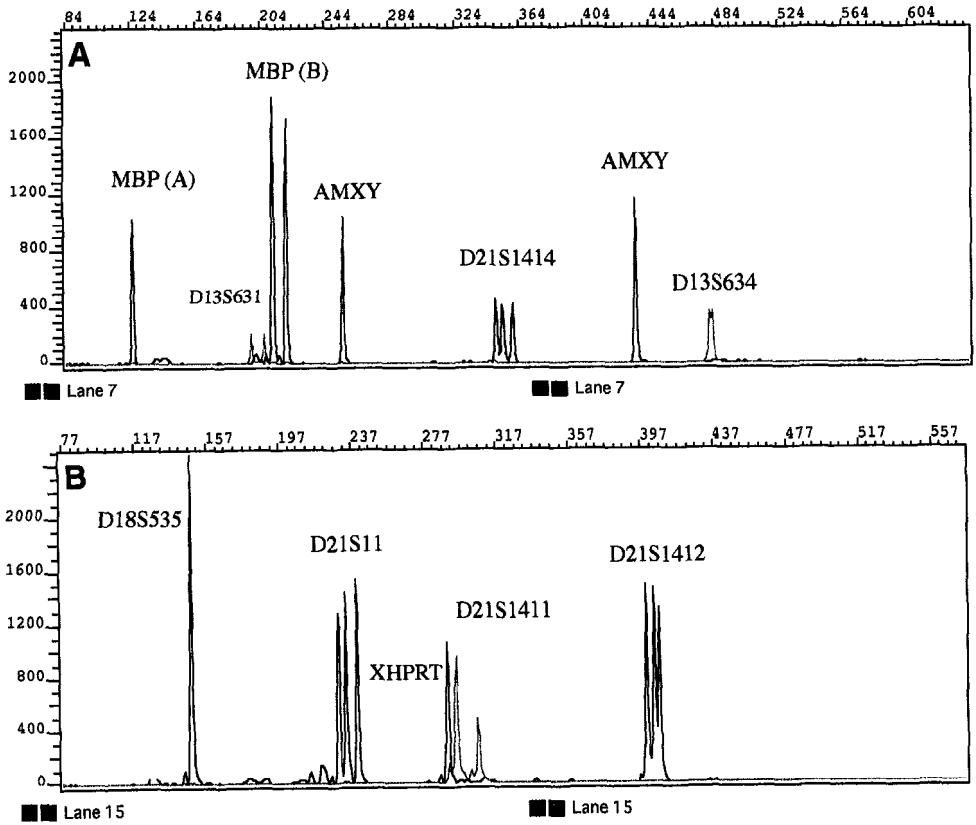

Fig. 2. **(A)** Electrophoretogram of the amplification products from a trisomy 21 sample. This sample displays a di-allelic trisomic pattern for the chromosome 21 marker D21S1414, the two alleles are in a 2:1 dosage ratio. A normal heterozygous pattern can be observed with the markers specific for chromosome 18 and D13S634. A homozygous pattern is observed with D13S631. Amplification of the marker AMXY results in one peak at 433 bp, corresponding to the X chromosome, and in another peak at 250 bp corresponding to the Y chromosome. **(B)** Electrophoretogram from the same trisomy 21 sample as shown in (A). This sample displays a tri-allelic pattern for the chromosome 21 markers D21S1411 and D21S1412 and a di-allelic trisomic pattern for the chromosome 21 marker D21S11.

fication using two STR markers specific for chromosome 21 (D21S11 and D21S1414), STR markers for two loci on chromosome 18 (MBP Locus A and B), and primers for the amelogenin regions of chromosomes X and Y. In this study, only one out of 76 samples tested showed an abnormal STR amplification pattern. When a chromosome 21 trisomic sample was tested, a

"diallelic" trisomic pattern was observed with the D21S11 and D21S1414 markers, but the chromosome 18 specific MBP marker produced a ratio of 1.6 between the two peak areas at locus A whereas a normal 1:1 ratio was observed at the locus B.

The aim of the third study was to extend and improve the diagnostic power of this molecular procedure for the simultaneous detection of aneuploidies involving chromosomes 21, 18, 13, and X *(12)*. Two multiplex PCR assays were designed for amplification of four STR markers specific for chromosome 21, two markers for chromosome 18, two markers for chromosome 13, one marker for chromosome X, and one marker for the amelogenin regions of both gonosomes (**Tables 1** and **2**). This method allowed the correct detection of trisomies 21, 18, 13, and triploidies, while sexing was performed simultaneously by amplification of DNA sequences derived from the X and Y chromosomes. Three out of 85 samples tested produced unsatisfactory results when tested with one of the two chromosome 13 specific markers. In these three cases, the amplification of the other chromosome 13 marker always resulted in a correct normal pattern, thus emphasizing the need for using more than one specific marker for each chromosome. In this study, all samples were heterozygous, at least for one marker specific for chromosome 21, and, thus informative. The chromosome 18 specific markers were uninformative (monoallelic) in two out of 85 samples, the chromosome 13 specific markers were uninformative in another two cases.

The results of these three studies demonstrate the diagnostic value of the QF- PCR techniques. However, in view of its targeted aim this prenatal test must be used in conjunction with conventional chromosome analysis in order to detect other chromosomal disorders. Since this assay can also be performed on very small samples or even on single cells *(10)*, it could be applied for prenatal diagnosis using blastomeres from preimplantation embryos or single cells isolated from peripheral maternal blood *(15–17)* and transcervical samples *(18–19)*.

2. Materials

2.1. Isolation of DNA

Molecular biology grade reagents and sterile distilled deionized water are used for all solutions.

1. Source of tissue (fetal liver, skin, muscle, chorionic villi) or cells (blood, amniotic fluid) from which DNA will be extracted (*see* **Notes 1–3**).
2. TE buffer, pH 7.5: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* ethylenediamine tetra-acetic acid (EDTA).
3. Proteinase K: 10 mg/mL in distilled water; store at –20°C.
4. 10% sodium dodecyl sulfate (SDS): (w/v) in deionized water, filter-sterilized.
5. CVS buffer: 150 m*M* NaCl, 25 m*M* EDTA pH 8.0; store at 4°C.

Table 1
STRs and Primers Used for the Detection of Trisomies 21, 18, 13, and Sexing (Set 1)

Marker	Sequence of the primers	Chromosome location	Heterozygosity	Refs	Optimal amount, pmol	Sizes of PCR products, (bp)	Label
D21S1414(F) D21S1414(R)	5'-aaa-tta-gtg-tct-ggc-acc-cag-ta-3' 5'-caa ttc ccc aag tga att gcc ttc-3'	chrom 21	0 88	Genome Data Base (Gerken, personal Communication)	2.5	330–380	5'-FAM
MBP(F) MBP(R)	5'-gga cct cgt gaa tta caa tc-3' 5'-att tac cta cct gtt cat cc-3'	18q23–qter	Locus A 0 80 Locus B 0 79	Polymeropoulos (1992) *(14)* Boylan 1990 *(15)*	20	Locus A 122–124 Locus B 208–232	5'-FAM
D13S634(F) D13S634(R)	5'- tcc aga tag gca gat tca at-3' 5'-cct tct tct tcc cat tga ta-3'	13q14 3–22	0 81	Genome Data Base	20	375–	5'-HEX
D13S631(F) D13S631(R)	5'-ggc aac aag agc aaa act ct-3' 5'-tag ccc tca cca tga ttg g-3'	13q31–32	0 94	Genome Data Base	2 5	209–	5'-HEX
AMXY(F) AMXY(R)	5'-ctg atg gtt ggc ctc aag cct-3' 5'- atg agg aaa cca ggg ttc ca-3'	X and Y		Modified after Nakahori et al (1991) *(16)*	2.5	X 432 Y· 250	5'-FAM

Table 2
STRs and Primers Used for the Detection of Trisomies 21, 18, 13, and Sexing (Set 2)

Marker	Sequence of the primers	Chromosome location	Heterozygosity	Refs	Optimal amount, pmol	Sizes of PCR products, (bp)	Label
XHPRT(F)	5'-atg cca cag ata ata cac atc ccc-3'	Xq 26 1	0 73	Edwards (1992) *(5)*	5	260–302	5'-FAM
XHPRT(R)	5'-ctc tcc aga ata gtt aga tgt agg tat-3'			EUROGEM linkage map *(17)*			
D18S535(F)	5'-tca tgt gac aaa agc cac ac-3'	18q12 2–12 3	0 92	Genome Data Base	5	138–	5'-FAM
D18S535(R)	5'-aga cag aaa tat aga tga gaa tgc a-3'						
D21S1412(F)	5'-cgg agg ttg cag tga gtt g-3'	Chrom 21	0 80	Genome Data Base	5	305–	5'-FAM
D21S1412(R)	5'-ggg aag gct atg gag gag a-3'	(S165–S198)		Utah Marker Development Group *(18)*			
D21S1411(F)	5'-atg atg aat gca tag atg gat g-3'	21q22.3	0.93	Genome Data Base	17 5	239–	5'-HEX
D21S1411(R)	5'-aat gtg tgt cct tcc agg c-3'	(S171–S198)		Utah Marker Development Group *(18)*			
D21S11(F)	5'-tat gtg agt caa ttc ccc aag tga-3'	21q21	0.93	Sharma and Litt (1992) *(19)*	17.5	172–264	5'-FAM
D21S11(R)	5'-gtt gta tta gtc aat gtt ctc cag-3'						

6. Lysis buffer: 0 32 *M* Sucrose, 10 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 1% v/v Triton X-100, store at 4°C.
7. Phenol/chloroform/isoamyl alcohol (25:24:1)
8. 4 *M* NaCl.
9 70% Ethanol.

2.2. Performing QF-PCR

1 The oligonucleotide primers used for the different STR markers and for sexing are listed in **Tables 1 and 2**. The forward oligonucleotide primers for D21S11, D21S1414, D21S1412, XHPRT, AMXY, MBP, and D18S535 are 5' end-labeled with 5-carboxy-fluorescein (blue), whereas the forward primers for the D21S1411, D13S634, and D13S631 markers are labeled with 2', 7'-dimethyloxy 4', 5'-dichloro 6-carboxy-fluorescein (green). Store aliquots at –20°C (*see* **Note 4**).
2 *Taq* DNA polymerase may be purchased from several suppliers (e.g., Promega, Madison, WI).
3. *Taq* polymerase buffer supplied together with Promega *Taq* DNA Polymerase (10X buffer is 100 m*M* Tris-HCl, pH 8.8, 15 m*M* $MgCl_2$, 500 m*M* KCl, 1% Triton X-100) is used in our laboratory.
4. 5 m*M* Deoxynucleotide stocks (dATP, dCTP, dGTP, and dTTP), made up in sterile water Store at –20°C
5 Light mineral oil

2.3. Electrophoresis and Analysis of PCR Products Using the Model 373A DNA sequencer and Genescan 672 Software

1. The automated fluorescent DNA sequencer is the Applied Biosystems, (Foster City, CA) Model 373A.
2. 10X TBE: 0.89 *M* Trizma base, 0.89 *M* boric acid, 0.02 *M* EDTA. The pH should be approx 8.3 at room temperature.
3. SequaGel XR, composed of 19:1 acrylamide/*bis*acrylamide Store at room temperature in a cool, dark area.
4. 10% Ammonium persulfate: 10% (w/v) in deionized water. Always make up fresh.
5. Loading buffer: Add 0.5 g of dextranblue to 4 mL formamide and store at 4°C in a dark area.
6. Size standard GENESCAN 500-ROX
7. Glass plates. notched and plain plate, 24 cm.
8. A pair of spacers and 24-well gel casting comb

3. Methods

3.1. Isolation of DNA

The classical phenol/chloroform DNA extraction method can be used for the isolation of DNA for PCR from amniotic fluid, fetal blood, and tissue samples *(20)*.

1. For amniotic fluid samples. Spin 1.5 mL of uncultured amniotic fluid at 138*g* at room temperature for 3 min. Pipet off the supernatant and resuspend in 100 µL CVS buffer.
2. For CVS and tissue samples: Transfer up to 20 mg of the sample into a test tube containing 500 µL CVS buffer. Vortex to further break up the tissue.
3. Add 10 mg/mL proteinase K and 10% SDS. For amniotic fluid samples we add 5 µL 10% SDS and 2 µL proteinase K, for CVS samples we use 10 µL 10% SDS and 10 µL proteinase K. Mix and incubate at 37°C overnight or at 55°C for 1–2 h.
4. Extract the sample twice with an equal volume of phenol/isoamylalcohol/chloroform (1:1 by volume)
5. Extract twice with an equal volume of chloroform.
6. Add 2 vol of 100% ethanol and 1/10 vol of 4 *M* NaCl. Mix gently. The DNA should form a precipitate. If no precipitate is visible, leave at –20°C for 1 h before spinning.
7. Recover DNA by centrifugation at 13,000*g* for 10 min.
8. Rinse pellet with 70% ethanol, decant, and air dry.
9. Resuspend DNA in TE buffer.
10. For fetal blood samples: Mix fetal blood samples, which are generally small volume samples (0 3-1 mL), with a double volume of lysis buffer; then place the sample on ice for 5–15 min Pellet white blood cells at 2000 rpm for 20 min at 4°C Remove and discard supernatant. Resuspend cells in approx 100 µL CVS buffer Add 5 µL 10% SDS and 2 µL 10 mg/mL proteinase K. Mix and incubate at 37°C overnight or at 55°C for 1–2 h. Then follow amniotic fluid protocol for DNA extraction.

3.2. Performing QF-PCR

1. Carry out PCR amplification in a total volume of 25 µL containing 5 µL of extracted DNA, 200 µ*M* dNTPs, 5–20 pmoles of each primer (**Tables 1 and 2**), 1X *Taq* polymerase buffer (1.5 m*M* $MgCl_2$) and 1.5 U of *Taq* DNA polymerase
2. Two separate multiplex PCR assays were designed with the following markers:
   a. D21S1414, AMXY, MBP (Locus A and locus B), D13S631, and D13S634 (Table 1),
   b. XHPRT, D18S535, D21S1412, D21S1411, and D21S11 (**Table 2**).
3. The reaction mix is overlayed with sufficient mineral oil to prevent evaporation Transfer the tube to a thermal cycle and preheat at 95°C for 5 min. Hot start the reaction by addition of 1.5 U of *Taq* DNA polymerase (*see* **Note 5**).
4. Then immediately initiate the following program for 22–24 cycles: denaturation at 94°C for 48 s, primer annealing at 60°C for 48 s and primer extension at 72°C for 1 min Final extension is for 5 min at 72°C At the end of thermal cycling, the samples are kept at 4°C.

3.3. Electrophoresis and Analysis of PCR Products Using the Model 373A DNA Sequencer and Genescan 672 Software

1. A set of glass plates is used which gives an effective migration distance of 24 cm. The "outer" side of the plates may be marked on one corner by a glass etcher to maintain each plate with a designated "inner" side to avoid gel bubbles caused by small scratches.

2. Lay the unnotched plate on a flat stable platform Lay gel spacers along each long edge of the plate and place the notched plate over the unnotched plate and spacers. Clamp the sides of the plates.
3. Prepare the gel solution by adding 10 mL of buffer reagent to 40 mL of monomer solution (SequagelXR) for each 50 mL of total volume needed (1:4 ratio). Add 400 µL of 10%APS to the 50 mL gel solution in a 100 mL flask and gently swirl for a few s. Pour the gel solution between the plates along the notched edge of the top plate. Start at one side of the assembly and slowly move across the top, allowing capillary action to pull the solution into form Allow the gel to polymerize for at least 2 h (*see* **Note 6**).
4 Remove the clamps and casting comb. Rinse the outside surface of the plates with water and then with absolute ethanol and allow to air dry. Place the gel assembly into the electrophoresis chamber of the sequencer. Fill the upper and lower chamber with 1X TBE. Carefully remove the comb between the plates.
5. Pre-run the gel for 10–30 minutes before loading samples Constant power (27W) is kept throughout the pre-run and the analytical run
6. Combine 3 µL PCR product with 0.8 µL GENESCAN-500 ROX containing the reference molecular size standard and 2 5 µL loading buffer Heat for 5 min at 95°C. Carefully flush urea out of all sample wells using a Pasteur pipet. Load all samples.
7. Begin electrophoresis at 27W constant power Electrophoresis conditions are: 2500 V, 45mA and 40°C for 7 h.
8. At the completion of the run, the Macintosh computer will automatically start the Data Analysis program (*see* **Notes 7** and **8**).
9. Information of the analyzed data are displayed in four ways:
   a. In the Gel window as colored bands that represent unanalyzed DNA fragments
   b. As electrophoretograms that show analyzed fragments as colored peaks.
   c. As tabular data that show detailed quantitative information for each analyzed fragment.
   d. As combination of electrophoretograms and the corresponding tabulated data.
10. Evaluation of the fluorescent PCR products: In the electrophoretogram, the results are displayed as peaks of fluorescent intensities (y-axis) as they occur over time—as represented by the scan number (x-axis). If an internal size standard is run with the sample, peaks can be aligned by fragment sizes (instead of by scan numbers). The amplification products are analyzed for the number of alleles detected and for the quantitation of the peak area of each allele peak. Most normal samples will show two STR products with two peaks of similar fluorescent intensities and a dosage ratio of 1:1. Trisomic samples will show either 3 STR peaks with a ratio of 1:1:1 or 2 peaks with a ratio of 2:1. The relative peak areas are calculated by dividing the large peak area by the smaller one (*see* **Notes 9** and **10**).

4. Notes

1. Blood samples should be collected in tubes containing EDTA (1 mg/mL) as an anticoagulant. However, whole blood specimens are frequently collected in heparin

in a clinical setting and are the only material available. Heparin can cause attenuation or complete inhibition of target DNA amplification during PCR amplification *(21)* Incubation of the DNA with either heparinase I or II can abolish this effect. The protocol described by Izraeli et al. *(22)* can be used, based on the incubation of the heparin-contaminated DNA samples with about 0.1–1 U heparinase I per μg DNA (in heparinase buffer: 20 m*M* Tris-HCl, pH 7.5, 4 m*M* $CaCl_2$, 50 m*M* NaCl, 0.01% albumin) for 2 h at room temperature

2. An alternative DNA extraction method is the DNA extraction method with Qiagen
3. Because of the small quantity of material available for analysis, we do not measure the amount of DNA present in an amniotic fluid sample. The cell number of amniotic fluid samples is highly variable. Starting with a 1.5 mL amniotic fluid sample we dissolve the DNA pellet after precipitation of the DNA in 10–20 μL TE This DNA concentration yields a sufficient amount of PCR products for three to six PCR reactions with our protocol.
4. Primer: The STR markers described in **Tables 1** and **2** have a high polymorphism information coefficient. Thus, the incidence of homozygous and, therefore, uninformative STR pattern is low When four different STRs are employed for the detection of Down syndrome, the probability of homozygosity at all four loci is reduced to a minimum. Samples that are uninformative when tested with markers for a given chromosome can be retested seperately with other STRs specific for this chromosome. We are using the markers D183386 for chromosome 18, D135258 for chromosome 13, and DX5337 for the X-chromosome.
5. The hot start PCR, as described above, has not given any problems of cross-contamination. In general, the optimal procedures are those in which the tubes, once closed and heated, are not be opened until thermal cycling is complete. A good and safe way to perform a hot start is by physically separating the reaction components with various materials that melt at higher temperatures, such as paraffin or agarose *(23)*.
6. Incomplete polymerization of the gel is characterized by distorted patterns in the gel file. The time sufficient for polymerization is at least 2 h. Always use fresh 10% ammoniumpersulfate (APS). APS begins to decompose at once when dissolved in water which results in a loss of activity.
7. A trouble-shooting section for the computer and the 373A-system is provided in the manuals supplied with the system.
8. An ABI Prism Genetic Analyser (Perkin Elmer) can also be used to size and quantify DNA fluorescent fragments. Electrophoresis of the samples is performed sequentially in capillary tubes which are automatically filled with the gel, the samples to be tested, the buffer, and the size standards. Optimal electrokinetic injection time and electrophoretic running conditions can be modified to increase the accuracy and precision of the tests.
9. Sometimes, one of the primers of the multiplex PCR reaction produces an equivocal result. This marker should be retested in a separate reaction. The marker most prone to abnormal and less precise STR pattern is the Locus A of the MBP marker

specific for chromosome 18 as already described by Mansfield *(8)*. For this reason we often have to repeat the MBP marker separately. If the abnormal pattern of the Locus A of the MBP marker remains, we consider this result as uninterpretable and rely solely on the STR results of the Locus B of the MBP marker and the third chromosome 18 specific marker D18S535.

10. The optimal fluorescent intensity of the PCR products should be about 5000 to 15000 U. In case of higher fluorescent intensities, there may appear background noise containing several extra peaks which make the interpretations of the STR pattern very difficult. To eliminate these effects, dilutions of the PCR products should be made and reloaded onto the gel. If background noise still occurs, PCR amplification should be repeated with fewer cycles.

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24

Detection of the Hepatitis C Virus by RT-PCR

Christopher John Healey and Steven Read

1. Introduction

The hepatitis C virus (HCV) is an organism of the age of molecular biology, for its discovery and much of the research into the infection have relied heavily on molecular techniques. The development of molecular cloning enabled a successful strategy that finally identified HCV *(1)* as the cause of 90% of post-transfusion *(2)* and > 50% of sporadic non-A, non-B hepatitis *(3)* after the failure by immunological techniques to discover the responsible agent. It is an important infection as most infected patients developed chronic hepatitis (> 50%) that can progress to cirrhosis and hepatocellular carcinoma *(4–6)*. Following the identification of the viral genome, antibody tests were developed which could detect exposure to the virus *(7)*. The presence of antibodies to HCV, however, does not distinguish between those with chronic infection and those who had cleared the virus. Chronic HCV infection can be difficult to diagnose as patients may be asymptomatic and have normal liver biochemistry *(8,9)* despite abnormal liver histology. Therefore, demonstration of virus RNA (usually from serum samples) is often necessary to confirm infection. Detection of HCV RNA requires the sensitivity of nucleic acid amplification (e.g., the polymerase chain reaction) as circulating levels of virus RNA can be very low *(10,11)*. Such tests are now widely used to confirm infection, monitor the response to anti-viral therapy, and in epidemiological studies of HCV infection.

Hepatitis C is a single-stranded RNA virus of the flaviviridae group that includes yellow fever virus and dengue viruses and is also closely related to the pestiviruses e.g., hog cholera virus *(12)*. The virus demonstrates considerable heterogeneity and it is possible to classify the virus into at least six major genotypes by comparative sequence analysis *(13)*. There are also differences in the degree of variation across the viral genome, e.g., hypervariable

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regions can be demonstrated in the areas that encode potential glycoproteins of the viral envelope *(14)*. The genome contains a large open reading frame that encodes a poly protein precursor that is preceded by the 5'-non-coding region (5' NCR). As the 5'NCR represents the most highly conserved region of the genome, it has proved an ideal site for amplification by the polymerase chain reaction (PCR) *(15,16)*.

The detection of HCV RNA involves several steps. First, viral RNA must be extracted from blood, concentrated, and made available for amplification through reverse transcription to a complementary DNA (cDNA) copy of itself. This cDNA is used as the template for amplification by PCR. Although PCR is intrinsically highly sensitive, it is usually necessary, when amplifying RNA viruses, to use a "nested PCR" protocol, i.e., two consecutive rounds of amplification with the second using an inner primer set to amplify the first round product. The multiple steps of this protocol increase the risks of pipeting errors, degradation of the sample, and contamination. However, it is possible to adjust the conditions so that the cDNA synthesis and first round PCR are performed in a single reaction using the outer set of primers to prime both the reverse transcription and PCR, thus reducing the risk of contamination *(17)*. This reduces the total time taken by the procedure. We describe our current protocol in the following steps: sample collection, RNA extraction *(18)*, combined RT/first round PCR, second round PCR, and detection. The sensitivity of the PCR means that contamination can occur easily, therefore, we follow the recommendations of Kwok and Higuchi to reduce this risk *(19)*.

2. Materials

2.1. RNA Extraction

1. Lysis buffer: Add 250 g guanidinium isothiocyanate (G9277 Sigma, St. Louis, MO) to 200 mL of 0.1 *M* Tris-HCl, pH 6.4, 44 mL 0.5 *M* ethylenediamine-tetra acetic acid (EDTA) pH 8.0, and 2.5 mL Triton X-100 (T8787, Sigma) (*see* **Note 2**). Stable at room temperature.
2. Silica suspension: Add 60 g silicon dioxide (S5631, Sigma) to 500 mL sterile distilled water in a measuring cylinder. Allow to settle for 24 h. Carefully remove 430 mL of supernatant and resuspend pellet in 500 mL distilled water. Stand for 5 h and then remove 440 mL supernatant. Add 600 µL concentrated HCl to pH 2 0. Aliquot, autoclave, and store at –20°C.
3. 1-mL Plastic Pasteurs and 1.5-mL Eppendorf pipets
4. Ethanol and acetone.

2.2. Combined RT/First Round PCR

2.2.1. Amplification Reagents

1. Primers (20):
   a. External pair:

5' CCC TGT GAG GAA CTW CTG TCT TCA CGC
GGT GCA CGG TCT ACG AGA CCT

b. Internal pair
5' TCT AGC CAT GGC GTT AGT RYG AGT GT
CAC TCG CAA GCA CCC TAT CAG GCA GT
W = A or T; Y = C or T; R = A or G

2 Thermostable DNA polymerase, (e.g., Red Hot DNA Polymerase, AB-0406, Advanced Biotechnologies, Epsom, Surrey, UK).
3. DNA polymerase buffer as appropriate to the chosen DNA polymerase.
4. Reverse transcriptase (Mu-MLV RT, AB-0322, Advanced Biotechnologies).
5 Deoxynucleotides (dNTPs) (10 m*M*).
6. Magnesium chloride (25 m*M*)
7. Light mineral oil (M3516, Sigma)
8. Tissue culture grade water (W3500, Sigma).
9 0.5-mL Eppendorfs and filter pipet tips.

2.2.2. Reagents for Detection

1 Agarose
2. TBE.
3 Ethidium bromide (Ethidium bromide is a mutagen and possible carcinogen and therefore should be handled at all times with gloves).
4 Loading buffer (50% glycerol, 0 5% bromophenol blue, 0.5% xylene cyanol).

3. Methods

3.1. Sample Collection

Samples should be collected in plain or EDTA tubes. HCV RNA is not stable and serum/plasma should be separated by centrifugation within 4 h *(11)*. Serum can then be frozen at –20°C for subsequent extraction and amplification, or more ideally for longer term storage at –70°C.

3.2. RNA Extraction

1. The RNA extraction should be performed in a clean fume cupboard as the lysis buffer can liberate cyanide gas. Add 100 µL serum to 1 mL extraction buffer and 40 µL silica suspension in a 1.5-mL labeled Eppendorf tube. Use appropriate positive and negative controls, including negative serum samples and water controls.
2. Gently vortex the sample and allow to stand at room temperature for 5 min and then spin for 10 s on high, i.e., 11,300*g* in a bench top microcentrifuge.
3. Carefully remove supernatant using a plastic Pasteur pipet and discard supernatant into 3 *M* NaOH (*see* **Note 2**).
4. Add 1 mL of ethanol (industrial methylated spirits will do) to each tube, vortex thoroughly to resuspend the silica pellet and spin on high as above Repeat this wash (**Steps 3** and **4**) with acetone instead of ethanol.

5. Remove acetone with plastic Pasteur pipet and evaporate off any excess acetone from the silica by incubating in a heating block for 5 min at 55°C with the tube tops open (*see* **Note 4**).
6. Add 50 µL sterile distilled water, vortex thoroughly and incubate at 55°C for 5 min with the tube tops closed. Vortex briefly again and spin for 1 min on high.
7 This supernatant can then be added to the prepared reaction mix (*see* **Subheading 3.3., step 4**). Avoid any carryover of silica (*see* **Note 5**), this may interfere with the subsequent PCR reaction.

3.3. Combined RT/First Round PCR

cDNA synthesis and the first round PCR are performed as a single reaction.

1. Combine the following reagents in a dedicated area, ideally, a laminar hood using dedicated equipment and reagents (*see* **Note 6**) Once reaction mix is made, remove from this area before the addition of the RNA extract
2. Combine the following reagents, sufficient for twelve reactions:
   60 µL of thermostable DNA polymerase buffer (10X buffer IV [without magnesium] Advanced Biotechnologies), 40 µL of 25 m*M* magnesium chloride (gives reaction concentration of 3 m*M* Mg^{2+}), 60 µL 10 m*M* dNTP mix, 200 µL sterile distilled water, 12 µL 5 µ*M* combined external primers (gives reaction concentration of 0 1 µ*M*), 1.5 µL Mu-MLV reverse transcriptase, and 1.5 µL (7 5 U) Red Hot DNA polymerase. Ensure that each constituent is well mixed (*see* **Note 7**)
3. Aliquot 30 µL of mix into prelabeled 0.5 mL Eppendorfs, and overlay each aliquot with 40 µL of light mineral oil.
4. In a separate area, carefully add 20 µL of RNA extraction below the oil layer using filter tipped pipets.
5. RT-PCR can then be performed with the following thermal profile: Reverse transcription—one cycle of 37°C 15 min, 94°C 2 min First round PCR—35 cycles of 94°C 30 s, 60°C 20 s, 72°C 30 s (*see* **Note 8**)

3.4. Second Round PCR

1. Combine the following reagents (for 12 reactions) under the same conditions outlined above—30 µL of thermostable DNA polymerase buffer (10X), 20 µL of 25 m*M* magnesium chloride (gives reaction concentration of 1 5 m*M* Mg^{2+}), 30 µL 10 m*M* dNTP mix, 210 µL sterile distilled water, 6 µL combined internal primers (gives reaction concentration of 1 µ*M*), and 1 5 µL (7 5 U) Red Hot DNA polymerase Ensure that each constituent is again well mixed.
2. Aliquot combined reagents into 24 µL volumes in prelabeled 0.5-mL Eppendorfs and overlay each reaction with 35 µL light mineral oil. Remove from the reaction mix preparation area and then transfer 2 µL of the amplified first round PCR product to the second reaction mix using a filter pipet tip (*see* **Note 9**).
3. Thermal profile for the second PCR—35 cycles of 94°C 20 s, 60°C 20 s, 72°C 30 s (*see* **Note 10**).
4. Detect and identify the final reaction product by electrophoresing 10 µL of the second round mix with 2 µL of loading buffer through a 2% agarose/0.5% TBE

gel containing ethidium bromide (0.5 μg/mL) and visualized under UV transillumination (*see* **Note 11**) The external and internal products are 299 and 235 base pairs long, respectively.

4. Notes

1. We have found that multiple freeze thaw cycles lead to the loss of RNA and, therefore, if multiple tests are to be performed on a single sample, aliquots for PCR should be stored separately Consequently, modern freezers with automatic defrost are inappropriate for storage of such samples.
   Care should be taken throughout this stage to prevent contamination; this should include frequent changing of gloves and the use of reagents dedicated to this procedure.
2. The guanidinium isothiocyanate acts as a strong protease, denaturing any potential RNase activity and the Triton-X100 helps to liberate HCV from its lipid envelope *(21)* Discarding the supernatant into strong alkali reduces the chance of liberation of cyanide fumes. The 3 *M* NaOH should be discarded appropriately when it has been diluted 10 times. When learning the technique, the number of control tubes should be increased to ensure no carryover from samples and no contamination in the reagents
3 It is possible to prepare 12 samples including any controls within 45–60 min with this method The preparation of the reaction mix can be carried out during the two incubations provided the reagents are already defrosted.
4. Air drying only of the remaining acetone before the addition of the water may increase recovery of RNA from the silica.
5 A second 50 μL aliquot of water can be used to extract remaining RNA from the silica. We have not found it necessary to use an RNase inhibitor when using this extraction method, however, the use of very pure water (RNase free) for resuspension is important.
6. The physical separation of the areas for RNA extraction and preparation of the PCR reaction mix, and the use of dedicated equipment and reagents greatly reduces the risk of contamination.
7. We have found that using a DNA polymerase (e.g , Red Hot DNA polymerase, Advanced Biotechnologies) that contains a colored indicator enables one to ensure adequate mixing of the reaction mix before aliquoting. This is important as both the enzymes are supplied in glycerol and will sink to the bottom of the tube. We have found it convenient to combine left and right hand primers of each set and store as aliquot stock solutions (5 μ*M*) Complete reaction mixtures (including enzymes) may be made up in advance, aliquoted and stored at –20°C. We have found these to be stable for at least 1 mo
8. The thermal profiles given are optimized for an Omnigene thermocycler and takes in total approx 2 h and 30 min for the combined RT and PCR step. Other thermal cyclers may require different temperatures/times for optimum performance with the primers described here.
9. After addition of 2 μL of the first round product to the second mix, freeze the remainder at –20°C. Subsequent positive samples can be amplified in bulk to

allow further analysis, e.g., restriction enzyme digestion for genotyping of the virus isolate *(22)*

10. The second reaction takes approx 2 h, 15 min. Altogether it is perfectly possible to complete all stages of the method within one working day.
11. Wear gloves at all times during the gel electrophoresis to avoid handling the ethidium bromide. Wear UV protective goggles during transillumination. When performing nested PCR with the primers described here, we have not found it necessary to perform hybridization on the PCR product.

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25

The Application of PCR to the Detection of *M. tuberculosis* in Sputum Samples

Majella Maher, Maura Glennon, Martin Cormican, and Terry Smith

1. Introduction

Human tuberculosis (TB) resulting from *Mycobacterium tuberculosis* and occasionally *Mycobacterium bovis* infection had been in decline for many decades, in the western world, owing to the advent of effective anti-microbial therapies and good public health programs that have controlled the spread of the disease. Nonetheless, there are currently in excess of one billion individuals infected with *M. tuberculosis*. TB accounts for approx 25% of all preventable deaths worldwide, resulting in more than 3.5 million deaths annually *(1)*.

Presumptive diagnosis of TB can be made based on patient history and radiological findings, but the isolation of *M. tuberculosis* is required for definitive diagnosis. At present, the laboratory diagnosis of TB relies mainly on microscopic examination or smears by the Ziehl-Neelsen (ZN) or Auramine-Rhodamine methods, and on the isolation of *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. microti and M. africanum*) organisms by culture. Direct microcopy is insensitive, requiring at least 10^3 organisms/mL for detection *(2)*. Microscopic examination does not permit distinction between *M. tuberculosis* complex organisms and other mycobacteria that may present a problem for diagnosis in some areas. Conventional culture can take up to six weeks depending on the system used *(3)*. The BACTEC radiometric system is a rapid culture method yielding results within 7–14 d. A number of novel commerical systems that are comparable to the BACTEC system, in terms of time to positive culture and do not require radioactivity, have recently become available.

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Table 1
Nucleotide Sequence of the Primers and Probe used in this Assay System

Primer and probe	Sequence
MBT3, 19 bases	5'-CCCAACTGGTGGGGCGTAG-3'
MB23S, 21 bases	5'-CTCGATGCCAAGGCATCCACC-3'
MB23S-MBT5; 40 bases	5'-CTCGATGCCAAGGCATCCA CCCAGTTCTCAAACACCACAC-3'
MB2; 20 bases	5'-GCATCAATGGATACGCTGCC-3'

Technological advances in the in vitro amplification of specific regions of bacterial DNA or RNA have led to the development of methodologies that facilitate the sensitive detection of microorganisms in clinical samples. The direct identification of mycobacteria in clinical samples using polymerase chain reaction (PCR) amplification offers *(4)* the potential for the same day diagnosis of infection *(5)*. Several groups have described PCR based assay systems for the sensitive and specific detection of *M. tuberculosis* complex organisms in clinical samples *(6–8)*.

The PCR assay for *M. tuberculosis* described in this chapter targets the amplification of a 298-bp fragment of the 16S/23S rRNA intergenic spacer region of members of the *M. tuberculosis* complex using an *M. tuberculosis* complex specific primer set MBT3/MB23S (*see* **Table 1**). The assay incorporates a 192-bp internal standard control (ISC) that may be coamplified with the MBT3/MB23S primer set, thereby monitoring potential PCR inhibition within each specimen (*see* **Fig. 1**). An *M. tuberculosis* complex specific oligonucleotide probe MB2 (*see* **Table 1**), increases the detection limit while adding a second level of specificity to this assay system *(9)*.

The assay involves three stages—sample preparation, PCR amplification, and product detection. This chapter describes the methods that will enable a laboratory to set up and perform this PCR based assay for the detection of *M. tuberculosis* in sputum specimens. Previous evaluation of the assay in our laboratory has proven it to be a sensitive and specific method for the detection of *M. tuberculosis* in sputum specimens *(10)*. It is important to remember that this PCR assay does not represent a stand-alone diagnostic tool, but must be considered in association with a patient's clinical history. While the sample preparation method outlined in this assay system has been used almost exclusively for sputum specimens, we have recently looked at other clinical specimens and found the method to be useful for some specimen types, e.g., semen and ovarian tissue samples allowing PCR amplification of bacteria present in these specimens.

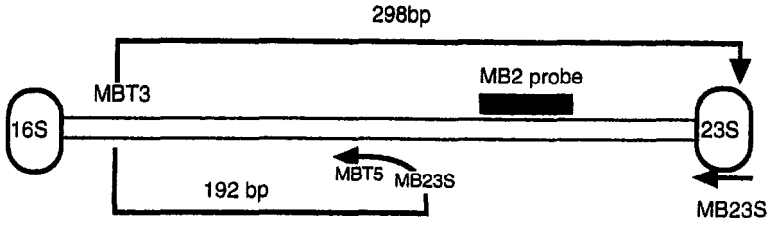

Fig. 1. The schematic representation of the position and design of the internal standard control for assessing PCR inhibition in clinical samples.

2. Materials

2.1. Lysis of Mycobacterial Cells

1. Sample lysis buffer (*see* **Note 1**): 100 m*M* NaOH, 5% Triton X-100, 15% Chelex (Bio-Rad Chelex 100 resin, Biotechnology Grade 100–200 mesh sodium form) 15% chelex should be added to the sample lysis buffer just prior to sample preparation and this aliquot of the buffer discarded after one sample preparation run
2. Sample neutralization buffer: 10 m*M* Tris-HCl, pH 7.5 prepared from a purchased stock of 1 *M* Tris-HCl, pH 7.5 (BDH) (*see* **Note 1**).

2.2. Purification of Genomic DNA

1. TE Buffer: 10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA.
2. CTAB/NaCl: Combine 4.1 g NaCl and 10 g CTAB (*N*-cetyl-*N.N.N.*-trimethylammonium bromide) and make up to 100 mL with dH_2O. This solution may need to be heated to 60°C to dissolve the CTAB.

2.3. PCR Amplification

1 10X PCR buffer (Promega [Madison, WI] or Gibco-BRL [Gaithersburg, MD]).
2. $MgCl_2$ (Promega or Gibco-BRL)
3. *Taq* Polymerase (Promega or Gibco-BRL).
4. 1.25 m*M* dNTPs are made up from 100 m*M* stocks (Promega) by combining 12.5 µL of each of dATP, dGTP, dCTP, dTTP, and bringing the volume to 1 mL with dH_2O.
5. 1.25 m*M* dU:dNTPs are made up from 100 m*M* stocks (Promega) by combining 12.5 µL of each of dATP, dGTP, dCTP, 4.5 µL dTTP, and 8.0 µL of dUTP. (Boehringer Mannheim, Indianapolis, IN), catalog number:104302) to make a 100 m*M* stock. Dissolve 9.36 mg in 200 µL dH_20. dNTP solutions can be prepared as larger stocks prealiquoted and stored at –20°C.
6. UNG-Uracil-*N*-glycosylase (Epicentre) may be purchased from Cambio Bio-Sciences, Cambridge, UK.
7. Proteinase K (Boehringer Mannheim).
8. Wizard PCR preps (Promega): For purification of ISC fragment.
9. Quick™Strips (Kodak): for quantification of purified ISC fragment.
10. Oligonucleotides (Genosys Biotechnologies, Cambridge, UK).

2.4. Detection of PCR Products

1. The DIG oligonucleotide 3' endlabeling kit (Boehringer Mannheim, catolog number: 1362372) contains all of the components needed to make a 3' endlabeled oligo probe with digoxigenin
2. The DIG Luminescent detection kit (Boehringer Mannheim, catolog number 1363514) contains all of the reagents required for chemiluminescent detection of digoxigenin-labeled nucleic acids The following additional reagents are required, but not supplied.
   a. Maleic acid buffer: 100 m*M* maleic acid, 150 m*M* NaCl; pH 7.5 (20°C)
   b. Washing buffer: (dilute 1·10 with H_2O) Maleic acid buffer (20°C) 0 3% Tween-20.
   c Blocking solution. 1% (w/v) blocking reagent for nucleic acid hybridization dissolved in maleic acid buffer. Blocking solution is cloudy and should not be filtered. It is stable for at least 2 wk when stored at 4°C, but then must be brought to room temperature before use.
   d. Detection buffer: 100 m*M* Tris-HCl, 10 m*M* $NaCl^+$, 50 m*M* $MgCl_2$; pH 9.5 (20°C).

3. Methods

We recommend that this assay be performed in three areas that are physically separated (preferably three separate rooms). Biologic materials must be handled according to good laboratory practice. All specimens should be handled as if potentially infectious *(11)*. A set of dedicated pipets should be made available for each stage of the assay and these should be used with plugged, aerosol free tips or positive displacement tips. During all stages of the assay, protective clothing should be worn (*see* **Note 2**).

3.1. Purification of M. tuberculosis Genomic DNA

The generation of a stock of internal standard fragment is achieved by PCR amplification of purified *M. tuberculosis* genomic DNA using the MBT3/MB23S-MBT5 primer set. The extraction of genomic DNA from *M. tuberculosis* cells as described by van Soolingen et al. *(12)* has been found to consistently yield high quality DNA for PCR amplification and consists of the following steps:

1. Resuspend the mycobacterial cells in 500 µL of TE buffer (*see* **Note 3**).
2. Heat at 80°C for 20 min.
3. Add 50 µL of 10 mg/mL lysozyme Incubate at 37°C for 1 h.
4. Add 70 µL of 10% SDS and 5 µL of proteinase K (10 mg/mL)
5. Incubate at 65°C for 10 min (*see* **Note 4**).
6. Add 100 µL of CTAB/NaCl prewarmed to 65°C, vortex until the liquid becomes white and incubate at 65°C for 10 min (*see* **Note 5**)
7. Add 750 µL of chloroform/isoamylalcohol and vortex for 10 s (*see* **Note 6**).
8. Centrifuge at 13000*g* for 5 min.

9 Transfer the aqueous phase to a fresh microcentrifuge tube.
10. Add 0.6 vol of isopropanol to precipitate the nucleic acid and place at –20°C for 30 min.
11. Spin at 13000*g* for 15 min and decant the supernatant.
12. Wash the pellet with 70% ethanol and decant
13. Dry the pellet at room temperature for 10 min.
14. Dissolve the pellet in TE buffer (*see* **Note 7**).

3.2. Design and Generation of Internal Standard Control Fragment (ISC)

When developing a PCR assay for application to clinical specimens, it is important to be able to determine whether a negative result reflects the absence of the target DNA (*M. tuberculosis)* or the presence of inhibiting substances. The addition of an artificially generated amplicon at a low level to the PCR reaction and coamplified with the same primers that amplify the target sequence will distinguish "true" negative results from "false negative" PCR reactions owing to presence of inhibitors in the lysed specimen. The ISC is added to the PCR reaction as a "PCRability" control, indicating that prepared sputum is not inhibiting the PCR amplification of the *M. tuberculosis* in the sample. The ISC for this assay system that is amplified by the MBT3/MB23S primer set (**Fig. 1**) was developed based on the work by Celi et al. (***9,13***). The ISC is generated by making an oligonucleotide 40 bases in length in which 20 nucleotides at the 3' end correspond to the opposite strand of a target sequence MBT5 (*see* **Table 1**) upstream from the MB2 probe system (**Fig. 1**). The MBT5 priming site is 133 bases from the end of the MBT3 primer sequence. The 20 nucleotides at the 5' end of the MB23S-MBT5 oligonucleotide corresponds to the sequence of MB23S. PCR amplification and purification of the ISC from *M. tuberculosis* genomic DNA consists of the following steps:

1. Add 30 ng (5 µL) of purified *M. tuberculosis* DNA to 95 µL of master cocktail containing a final concentration 100 m*M* KCl, 20 m*M* Tris HCl (pH 8.0), 4 m*M* $MgCl_2$, 1.25 m*M* dNTPs, 15 pmol of the *M. tuberculosis* complex specific primers MBT3/MB23S-MBT5, and 1.5 U *Taq* Polymerase (Promega).
2. Place in the thermocycler and enter the following program for 35 cycles: 95°C for 30 s, 55°C for 30s, 72°C for 30 s.
3. Post-PCR, run all of the PCR sample on a 1% low melting point agarose gel.
4. Excise the 192-bp fragment and purify using Wizard™ preps (Promega) according to manufacturer's instructions (see **Note 8**).
5. To estimate the concentration of purified ISC, serial dilutions should be run on a 2% agarose gel and compared to the size standard. The Quik™ strip from Kodak may be used to estimate the concentration of the ISC.
6. 3.5 fg of ISC is added into each PCR reaction.

3.3. Preparation of Sputum Specimens for PCR Amplification

The application of PCR to the detection of mycobacterial DNA in clinical specimens requires the preparation of specimens to make the DNA accessible for PCR. **Subheading 3.3.1.** outlines a recommended procedure for the concentration of mycobacteria from sputum specimens, whereas **Subheading 3.3.2.** describes a simple alkaline lysis of *M. tuberculosis* cells with a heat treatment step in the presence of chelex. Mycobacteria are considered difficult to lyse, and a variety of lysis methods have been published in the literature *(14–16)*. For routine processing of sputum samples, or for testing a large number of samples for the presence of *M. tuberculosis* it is important to choose a lysis method that is rapid and user friendly, with a minimal number of manipulations, to reduce the risk of cross contamination between samples. The lysis method we describe below fulfills these criteria.

3.3.1. Concentration of Mycobacteria from Sputum Specimens

All specimen manipulations should be performed in a Class 1 hood.

1. Add an equal volume of 1 *M* NaOH to the sputum specimen
2. Incubate the closed container with shaking for 30 min.
3. Neutralize the NaOH by the addition of 1 *M* NaH_2PO_4 to a final volume of 20 mL.
4 Centrifuge at 2500*g* for 15 min.
5. Decant the supernatant (*see* **Notes 9** and **10**).

3.3.2. Lysis of M. tuberculosis Cells in Sputum Specimens

All specimen manipulations should be performed in a Class 1 hood.

1. Remove sample preparation lysis buffer from the 4°C and allow it to come to room temperature.
2. Immediately before use, add 300 µL of sample preparation lysis buffer to 45 mg resin and mix thoroughly.
3 Vortex the sputum specimen and transfer 50 µL to a 1.5-mL screw cap tube or Eppendorf with a lid lock.
4. Centrifuge at 8500*g* for 10 min to pellet the cells.
5. Aspirate the supernatant and discard it.
6. Add 50 µL of sample preparation lysis buffer and preparation resin combined as per point *(2)* to the pellet.
7 Vortex to resuspend the pellet and incubate at 95°C for 45 min
8. After incubation, centrifuge briefly and add 50 µL of sample neutralization buffer Vortex to mix components (*see* **Notes 11** and **12**).

3.4. PCR Amplification of M. tuberculosis in Lysed Sputum Specimens

The PCR amplification of a 298-bp target from the 16S/23S rRNA intergenic spacer region of members of the *M. tuberculosis* complex and the coamplifica-

tion of the 192-bp ISC using the MBT3/MB23S primer set is described in this section. Each lysed sputum specimen is PCR amplified in duplicate with the ISC fragment added to one PCR reaction for each sample to monitor PCR inhibition within that specimen (*see* **Note 13**). The utilization of the enzyme Uracil-N-glycosylase (UNG) and the incorporation of dUTP in the preliminary step of the PCR amplification, prevents false positive results owing to the carryover of amplicons from a previous PCR amplification ***(17)*** (*see* **Note 14**).

1. Label two tubes per sputum specimen: one tube will have internal standard added; this will be referred to as the PCRability control test. The other tube will not have internal standard control included; this will be referred to as the sample test. Include a no template negative PCR control tube by adding 5 µL of H_2O in place of prepared sputum (*see* **Note 15**).
2. Add 5 µL of lysed sputum specimen to 95 µL of master cocktail containing a final concentration of 100 m*M* KCl, 20 m*M* Tris-HCl (pH 8.0), 0.2% Triton X-100, 6 m*M* $MgCl_2$, 1 25 m*M* dNTPs (dATP, dGTP, dCTP, dUTP/dTTP(2:1), 30 pmols of the *M tuberculosis* complex specific primer set MBT3/MB23S, and 2.5 U *Taq* Polymerase, and 1 U UNG (Sample Test) Overlay with 3 drops of mineral oil if necessary (if a PCR machine with a heated lid is not being used)
3. Add 5 µL of ISC fragment (3.5 fg) and 5 µL of lysed sputum specimen to 90 µL of master cocktail containing a final concentration of 100 m*M* KCl, 20 m*M* Tris-HCl (pH 8.0), 0.2% Triton X-100, 6 m*M* $MgCl_2$, 1.25 m*M* dNTPs (dATP, dGTP, dCTP, dUTP/dTTP (2:1), 30 pmols of the *M tuberculosis* complex specific primer set MBT3/MB23S, 2.5 U *Taq* Polymerase, and 1 U UNG. (PCRability control test). Overlay with 3 drops of mineral oil if necessary.
4. Place the PCR reaction tubes in the thermocycler.
5. Program the thermocycler as follows:

Step	Temperature/Duration	Cycles
Step 1	37°C for 10 min	1 cycle only
Step 2	95°C for 10 min	1 cycle only
Step 3	95°C for 30 sec	45 cycles
Step 4	69°C for 30 sec	45 cycles
Step 5	72°C for 1 min	45 cycles
Step 6	4°C	Hold indefinitely

3.5. Detection of the Amplified PCR Product

Recently, there has been extensive research in the development of user friendly detection formats incorporating nonradioactive technologies with colorimetric, fluorometric, or chemiluminescent endpoints ***(18–20)***. We describe a nonradioactive format using a DIG-labeled MB2 probe and chemiluminescent detection (Boehringer Mannheim). Post-PCR, a 20 µL aliquot of each PCR reaction is run on a 2% agarose gel. Samples with the ISC added should have a visible band at 192 bp indicating that the sample was amenable

to PCR. TB positive samples may have a visible band at 298 bp (*see* **Note 16**). The gel is Southern blotted *(21)* onto a nylon membrane, U.V. crosslinked and probed according to **Subheading 3.5.1.**, and detected as described in **Subheading 3.5.2.** The oligonucleotide probe MB2 does not hybridize to the ISC, hybridizing only with the amplified *M. tuberculosis* PCR product.

3.5.1. DIG Labeling and Hybridization with MB2

1. Add the following reagents in order to a microfuge tube on ice: 4 µL 5X rxn buffer, 4 µL $CoCl_2$, 100 pmol oligonucleotide, 1 µL Dig-ddUTP, 1 µL terminal transferase, H_2O to 20 µL.
2. Incubate the reaction at 37°C for 15 min. Place on ice.
3. Add 1 µL EDTA to stop the reaction (*see* **Note 17**)
4. Prehybridize the filter for 60 min at 45°C in a solution of 6X SSC, 10X Denhardt's, 0 1%SDS.
5. Add 10 µL of DIG labeled MB2 probe to 10 mL of hybridization solution (6X SSC, 0.1% SDS) and hybridize for 2 h at 45°C.
6. Wash the filter as follows:
   a. 2X SSC/0.1% SDS at 45°C for 10 min.
   b. 0.2X SSC/0.1% SDS at room temperature for 10 min
7. Proceed to detection (**Subheading 3.5.2.**).

3.5.2. Chemiluminescent Detection Procedure

1. After hybridization and stringency washes, rinse the membrane briefly (1–5 min) in washing buffer
2. Incubate for 30 min in 100 mL blocking buffer
3. Dilute anti-DIG-AP conjugate (vial 3) to 75 mU/mL (1:10000) in blocking buffer (1X conc.).
4. Incubate membrane for 30 min in 20 mL antibody solution.
5. Wash twice for 15 min with 100 mL washing buffer.
6. Equilibrate 2–5 min in 20 mL detection buffer.
7. Dilute CSPD (vial 5) 1:100 in detection buffer.
8. Incubate the membrane for 5 min in 10 ml CSPD solution.
9. Let the excess liquid drip off and blot membrane briefly (DNA side up) on Whatman 3MM paper. Do not let membrane dry completely
10. Seal damp membrane in a hybridization bag and incubate for 5–15 min at 37°C to enhance the luminescent reaction.
11. Expose for 15–20 min at room temperature to X-ray or Polaroid b/w film.

4. Notes

1. Lysis (without chelex) and neutralization buffer can be prepared as large stocks and stored at 4°C.
2. We would recommend that, if possible, the methods outlined in this chapter should be performed in biological cabinets that allow product protection, thereby, providing an additional safeguard against PCR contamination.

3. *M tuberculosis* is a hazardous organism and should only be cultured by appropriately trained laboratory personnel in a suitable containment facility. *M. tuberculosis* can be cultured in Middlebrook 7H11 broth with OADC supplement. The organisms are harvested for nucleic acid extraction by centrifugation Alternatively, they may be grown on solid media (Lowenstein-Jensen). A homogenous suspension for nucleic acid extraction can be prepared by transferring colonies to a capped tube with glass beads and vortexing for several minutes (in a safety cabinet). Allow the large clumps to settle out and process the suspension as for broth culture.
4. The solution should become viscous after detergent lysis of the bacterial cells
5. This step is very important since an *N*-cetyl-*N.N.N.*-trimethylammonium bromide (CTAB)-nucleic acid precipitate will form if the salt concentration drops below about 0 5 *M* at room temperature *(22)*. The aim here is to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB while retaining the nucleic acid in solution.
6. The chloroform/isoamyl alcohol extraction step removes the CTAB protein/polysaccharide complexes. A white interface should be visible after centrifugation
7. Redissolving the precipitate may take up to 1 h as the DNA is of high molecular weight.
8. The ISC fragment is electrophoresed and excised from a low melting point (LMP) gel to eliminate the possibility of *M. tuberculosis* genomic DNA getting into the ISC stock where it could lead to the generation of false positive results
9 Any method of liquefaction of sputum followed by centrifugation is suitable
10. The method described in **Subheading 3.3.1.** should be performed in a biological safety cabinet.
11. Sputum specimens should be stored at –20°C in 50-µL aliquots prior to sample preparation and returned to biological cabinet for lysis
12. Lysis of mycobacterial cells, using sample preparation method, should be performed just prior to PCR amplification to ensure sensitive amplification as storage of prepared sample for >1 h prior to PCR amplification significantly reduces the sensitivity of amplification.
13. As the ISC in this assay system is 192 bp and, therefore, smaller than the *M tuberculosis* target fragment (298 bp), it may compete with the larger target for amplification if, for example, there is a low number of *M. tuberculosis* cells in the lysed specimen, the ISC may be amplified in preference to the *M tuberculosis* target. By including the second PCR amplification (sample test) reaction where no ISC is added, the problem of competition is overcome.
14. UNG catalyzes the destruction of dUTP containing DNA, but not dTTP containing DNA. When dUTP is used in a predetermined concentration ratio with dTTP, the generation of a PCR product that is susceptible to destruction by UNG is facilitated. Deoxyuridine is not present in the microbial target DNA, but is always present in the PCR product. In the first cycle of PCR amplification, the target DNA and reagent mixture, which contain dUTP are heated to 37°C, allowing the destruction of any dUTP containing product. The UNG enzyme is then heat inactivated, thereby, preventing the UNG from destroying any "real" amplified product

15. The scientist performing this PCR amplification may wish to include a positive control in the reaction series. This may be achieved by the addition of 30 ng (5 µL) of *M tuberculosis* DNA to 95 µL of the master cocktail as described. It should be noted that the ISC, while serving as a "PCRability" control for each lysed sample, is also a positive control for the assay.
16. Lysed specimens that have a low number of *M. tuberculosis* cells may not have a visible band on gel electrophoresis However, on hybridization with MB2, a positive signal at 298 bp will be obtained for that sample.
17. Boehringer Mannheim recommends a purification protocol that can be used to remove unincorporated DIG from a DIG-labeled oligonucleotide prior to addition to the hybridization solution. However, we have found it unneccessary to purify the oligonucleotide prior to hybridization

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Microsatellite Analysis in Human Disease

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1. Introduction

Microsatellites (or simple sequence length polymorphisms [SSLPs]; and short tandem repeat polymorphisms [STRs]) are tandemly repeated arrays of short stretches of nucleotide sequences, typically $(dA\text{-}dC/dT\text{-}dG)_n$ where n is between 15 and 30 *(1,2)*. The human genome is estimated to contain approximately 12,000 $(dA\text{-}dC)n$ microsatellites with a polymorphic information content (PIC) > 0.5 *(3)* Further information on microsatellite organization, distribution, and origin can be obtained from a recent review *(4)*. For the purposes of this chapter, it suffices to state that owing to their high heterozygosity, Mendelian codominant inheritance, ubiquity through the genome, and ease of polymerase chain reaction (PCR) typability, microsatellites are the markers of choice in the construction of human (and other model organisms) linkage maps. For instance, a recent human microsatellite linkage map consists of over 5264 $(dA\text{-}dC)_n$ loci with a heterozygosity >70% distributed along the genome at an average interval of 1.6 centiMorgans (cM) *(5)*. This forms an invaluable resource for the genetic dissection of complex traits (e.g., type I diabetes).

Highly informative markers, present in high density and easily assayed, have obvious forensic potential. Microsatellites are excellent candidates for personal identification systems, and such evidence is admissible in courts of law in several countries. As markers, they are also important in the genetic dissection of the neoplastic state. The allelotyping of tumor DNA for identification of interstitial chromosomal deletions and amplifications (and subsequent identification of putative tumor suppressor genes and oncogenes respectively) is made more precise by the use of microsatellite PCR-based assays.

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Apart from their important role as genome markers, microsatellites are directly implicated in human disease (reviewed in **ref. 4**). Briefly, the expansion of microsatellites (usually trinucleotide repeats), a consequence of their inherent instability, has been implicated in the pathogenesis of various cancers, Fragile X syndromes, and neuromuscular degenerative disorders including Huntington's disease and myotonic dystrophy. In addition, mutations in a variety of DNA mismatch repair (MMR) genes manifest predisposition to the hereditary nonpolyposis colorectal cancer syndrome. Microsatellite PCR assays of these malignancies reveal generalized microsatellite instability, with aberrant sized bands visualized in tumor DNA compared with normal.

For genetic mapping experiments, microsatellites need only be amplified qualitatively so that the size of the two alleles can be determined. Qualitative PCR can be readily performed without the use of radioisotopes. In order to investigate the accumulation of deletions and amplifications during the development of a malignant disease, however, microsatellite assays must be quantitative as well as qualitative. Using the equipment available in most molecular biology laboratories, this can only be achieved using a radiolabeled oligonucleotide in the PCR reaction. The use of fluorescent approaches to this analysis is a specialized matter and is briefly discussed.

Microsatellite PCR analyses can be performed on a variety of clinical materials. We will outline protocols for the use of both macroscopic fresh tissue and microscopically dissected archival tissue in such analyses. In view of the technically more demanding requirements for quantitative microsatellite PCR analyses, optimization protocols for PCR are emphasized, as are protocols for the use of radioisotopes. Alternatives for the resolution and detection of the amplified fragments are also discussed. The concluding section briefly deals with issues related to microsatellite PCR: the genesis and effects of "stutter bands," the possibilities for automation in analysis and the use of specialised fluorescence approaches

2. Materials

2.1. DNA Extraction

2.1.1. DNA from Macroscopic Tissues

1. Extraction buffer: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* EDTA pH 8.0, 0.5% sodium dodecyl sulfate (SDS) (w/v).
2. SDS buffer: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* EDTA pH 8.0, 0.5% SDS (w/v), 100 m*M* NaCl.
3. 10 mg/mL RNAase A (Sigma, St. Louis, MO). Store at –20°C.
4. 20 mL/mL Proteinase K (Boehringer Mannheim, Indianapolis, IN). Store at –20°C.
5. Tris-buffered Phenol pH > 7.6 (Severn Biotech, Kidderminster, UK).

6. Chloroform.
7. 10 *M* Ammonium acetate.
8. Isopropanol.
9. 100 and 70% (v/v) ethanol.
10. 1X TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.
11. Plastic micropestles (BDH, London, UK).

2.1.2. DNA from Microdissected Tissue Sections

1. Hematoxylin and Eosin (H&E) stained 7 μ*M* thick sections.
2. Glass Pasteur pipet-plugged.
3. DNA extraction buffer: 100 m*M* Tris-HCl, pH 8.2, 1 m*M* EDTA, pH 8.0.
4. 20 mg/mL Proteinase K (Boehringer Mannheim, East Sussex, UK). Store at –20°C.

2.2. Microsatellite PCR

1 Ampli*Taq* DNA polymerase. Store at –20°C.
2. 10X PCR buffers· prepare six buffers.
   a 10X buffer 1: 100 m*M* Tris-HCl, pH 8.0, 500 m*M* KCl, 1% Triton X-100, 15 m*M* $MgCl_2$
   b. 10X buffer 2: 100 m*M* Tris-HCl, pH 8.0, 500 m*M* KCl, 1% Triton X-100, 30 m*M* $MgCl_2$
   c. 10X buffer 3· 100 m*M* Tris-HCl, pH 8.5, 500 m*M* KCl, 1% Triton X-100, 15 m*M* $MgCl_2$
   d. 10X buffer 4· 100 m*M* Tris-HCl, pH 8.5, 500 m*M* KCl, 1% Triton X-100, 30 m*M* $MgCl_2$
   e. 10X buffer 5. 100 m*M* Tris-HCl, pH 9.0, 500 m*M* KCl, 1% Triton X-100, 15 m*M* $MgCl_2$
   f. 10X buffer 6. 100 m*M* Tris-HCl, pH 9.0, 500 m*M* KCl, 1% Triton X-100, 30 m*M* $MgCl_2$
4. 100 m*M* Stock dNTP solutions each at pH 8.0 (Pharmacia Biotech, St. Albans, UK). Store at –20°C.
5. Oligonucleotide primers (Oswel DNA, Southampton, UK).
6. Mineral Oil (Sigma, St. Louis, MO).
7. Thin walled microamp 0.2-mL tubes (Costar, Cambridge, MA), autoclaved.
8. GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA)

2.3. Primer Radiolabeling

1. T_4 polynucleotide kinase (MBI Fermentas, Vilnius, Lithuania). Store at –20°C.
2. γ-ATP of activity >5000 Ci/mmol (Amersham International [5000 Ci/mmol] or ICN [7000 Ci/mmol]). Store at –20°C.
3. Kinase buffer: 0.5 *M* Tris-HCl, pH 7.6, 0.1 *M* $MgCl_2$, 50 m*M* DTT, 1 m*M* Spermidine-HCl, 1 m*M* EDTA, pH 8 0. Store at –20°C.
4. Sepahdex G-25 (Pharmacia, St. Albans, UK).
5. TNES buffer: 20 m*M* Tris-HCl, pH 7.5, 140 m*M* NaCl, 5 m*M* EDTA, pH 8 0, 0.1% SDS (w/v).

6 Siliconized glass wool (autoclaved).
7. 1-mL Sterile disposable syringe
8. 50-mL Sterile Falcon tube.

2.4. Post-PCR Fragment Resolution

2.4.1. Nondenaturing PAGE

1. 40% Acrylamide/*bis* acrylamide 19·1 ratio (Sigma) (*Neurotoxin*)
2 *N.N.N'.N'*-tetramethylethylenamide (TEMED) (Sigma).
3. 10% Ammonium persulfate (w/v) (BioRad, Hercules, CA).
4. Dimethyldichlorosilane solution or Sigmacote (Sigma).
5. pBR322 DNA-*Msp* I Digest Molecular Weight Markers (New England Biolabs, Beverly, MA).
6. 1X TBE buffer: 90 m*M* Tris-borate, 2 m*M* EDTA
7. 6X Loading dye: 0.25% bromophenol blue (w/v), 0 25% xylene cyanol FF (w/v), 30% glycerol in water (v/v)
8. 50-mL Sterile syringe.
9. 19 gage needle
10. Protean II xi Cell PAGE apparatus with 20 cm long gel plates (Bio-Rad).

2.4.2. Denaturing PAGE

1. Sequagel acrylamide kit (Flowgen, Sittingbourne, UK). We find this urea polyacrylamide premix the easiest and safest to use.
2. *N N. N'. N'*-tetramethylethylenamide (TEMED) (Sigma)
3 Ammonium persulfate 10% (w/v) (BioRad).
4. Dimethyldichlorosilane solution or Sigmacote (Sigma).
5. Liquid detergent.
6. 100% Ethanol.
7. 1X TBE buffer: 90 m*M* Tris-borate, 2 m*M* EDTA, pH 8.0.
8 Running dye: 98% Formamide (v/v), 0.025% bromophenol blue (w/v), 0.025% xylene cyanol FF (w/v), 10 m*M* EDTA pH 8.0
9. Sequencing ladder: M13 phage positive control (Sequenase Vers 2.0 DNA sequencing kit; Amersham, Little Chalfont, Bucks, UK)
10. 21 × 50 cm Sequi-Gen Sequencing Cell (BioRad) and electrophoresis power pack.

2.5. Detection of PCR Products

2.5.1. Ethidium Bromide Staining

1. Ethidium bromide stock solution 10 mg/mL (*Mutagen*). Store wrapped in foil at 4° C.
2 1X TBE buffer· 90 m*M* Tris-borate, 2 m*M* EDTA.

2.5.2. Silver Staining

1. Silver stain Plus Kit (Bio-Rad). We have obtained our best silver staining results with this kit. Staining is performed, essentially, to the manufacturer's instructions.

2. Fixative solution: 50% methanol (v/v), 10% glacial acetic acid (v/v), 10% fixative enhancer (v/v) (from kit).
3. 5% Glacial acetic acid (v/v).
4. Sterile water

2.5.3 Autoradiography

1. Fixative solution. 15% methanol (v/v), 5% acetic acid (v/v).
2. Whatman 3 MM paper.
3. Saran wrap (Clingfilm).
4. Vacuum gel dryer (BioRad).
5. Sensitize Preflash apparatus (Amersham).
6. Autoradiograph film (Kodak XAR5).
7. Autoradiograph cassette with intensifying screens (Kodak, Hemel Hempstead, Herts, UK).

3. Methods

3.1. DNA Extraction

In the clinical situation, a variety of human samples can be used as sources of template DNA for microsatellite PCR. For most fresh tissue or blood, standard extraction protocols with proteases and phenol-chloroform extraction, followed by precipitation of high molecular weight genomic DNA in high salt buffers with ethanol are suitable. Commercial kits offer convenient alternatives, minimizing handling and exposure to toxic chemicals, offset by a higher cost. However, DNA may need to be extracted from a variety of less suitable sources, including formalin fixed paraffin embedded tissue blocks, cytological smears, and cellular aspirates. Template DNA from such sources is often of variable quality (being extensively nicked) and low copy number. Such degraded DNA limits the size of the PCR fragment consistently amplifiable (below 400 bp for paraffin processed material).

In the subsequent section, two protocols are described. One for the extraction of microgram quantities of high molecular weight (HMW) genomic DNA from macroscopic frozen samples using phenol-chloroform, and the other, a specialized protocol for extraction of nanogram amounts of DNA from microdissected cell populations in archival H&E stained tissue sections.

3.1.1. DNA Extraction from Macroscopic Samples

A protocol for the extraction of high molecular weight (HMW) genomic DNA from fresh tissue samples is detailed. Many similar protocols with slight modifications exist (in addition to commercial kits). We find that the two rounds of phenol extraction in this protocol minimize protein contamination of the DNA, enabling reproducible microsatellite PCR.

1. Snap freeze 20–25 mg of tissue in liquid nitrogen or on a dry ice/ethanol bath
2. Transfer the tissue to a sterile 1.5 mL Eppendorf and homogenize the tissue with a plastic Micropestle in 750 µL of extraction buffer with 20 µg/mL of pancreatic RNAase (*see* **Note 1**).
3. Incubate at 37°C for 30 min.
4. Add proteinase K to 100 µg/mL and incubate at 52°C overnight. There should be no obvious tissue debris remaining after incubation
5 Add 500 µL buffered phenol, invert 20 times and centrifuge in a benchtop microfuge at 13,000 rpm (i.e., maximum) for 5 min Transfer supernatant (avoiding protein precipitates at the interface) to a fresh Eppendorf.
6. Repeat phenol extraction twice more, followed by extraction once with phenol/chloroform (1:1) and finally with chloroform
7. Add 0.2 vol (final) of 10 *M* ammonium acetate. Precipitate the DNA by adding 600 µL ice-cold 100% ethanol and incubating at –20°C for 10 min.
8. Spin the Eppendorf at 13,000 rpm (maximum) for 10 min A whitish DNA pellet should be visible
9 Discard the supernatant and wash the pellet with 500 µL of 70% ethanol. Spin at 13,000 rpm (maximum) for 1 min and discard the ethanol
10. Resuspend the pellet in 400 µL of SDS buffer
11. Perform a phenol extraction as above, followed by phenol.chloroform (1·1), and then chloroform extractions.
12. Precipitate the DNA by adding 500 µL isopropanol to the supernatant and incubating at –20°C for 10 min.
13. Spin the Eppendorf at 13,000 rpm (maximum) for 10 min. A translucent DNA pellet should be faintly visible.
14. Discard the supernatant and wash the pellet with 500 µL of 70% ethanol Spin at 13,000 rpm (maximum) for 1 min and discard the ethanol.
15. Vacuum dry the pellet and resuspend the dried pellet in 100 µL 1X TE solution. Solubilize the DNA at 4°C overnight or at 37°C for 2 h
16. Keep the DNA at –20°C for long term storage. The DNA can be quantitated by spectrophotometry, and the quality assessed by running it out on 0.7% agarose gel with size markers.

3.1.2. DNA from Archival Microdissects

We describe a protocol that we have used successfully in many tissue types to extract nanogram quantities of DNA from archival H&E-stained tissue sections. Sufficient DNA for multiple PCR analyses is obtained from minute tissue areas (Fig. 1).

1. Prepare plugged glass Pasteur pipets for microdissection: Holding both ends, heat the narrow part (approx 1 in. from the taper) in the colorless outer flame of a bunsen. Keep rolling the pipet to heat the glass evenly When the hot glass softens and bends, pull out the end several feet with a quick smooth movement When done correctly, a narrow hairlike glass capillary will be left connecting the two

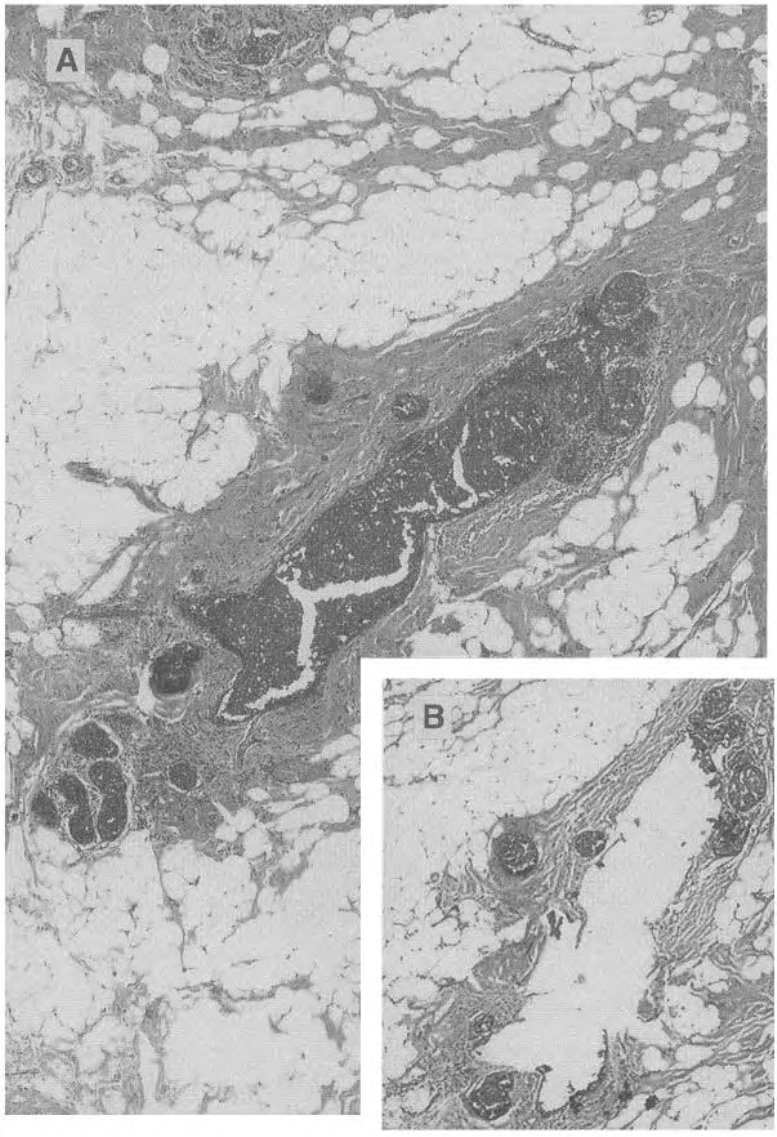

Fig. 1. Photomicrograph of an H&E stained tissue section from a case of breast cancer. *Inset*: a serial section of the same case showing the tumor focus microdissected.

ends. Break the capillary 1 in. from the point of formation. Store the pipets in a clean box on a bed of tissue paper (*see* **Note 2**).

2. Set up a microscope in a clean area and clean the microscope stage with 70% ethanol.
3. Dip an H&E-stained tissue section (without a coverslip) in 100% ethanol and place on the microscope. Quickly scan the slide under the microscope and determine a region of interest (using the 10 × and 40 × objective). Let the ethanol evaporate.
4. Take a prepared glass pipet and connect the plugged end to a length of plastic tubing. Using mouth suction at the other end of the tubing, suck up a minute quantity of sterile 20% ethanol through the capillary end.
5. Viewing through the microscope, carefully bring the tip of the hairlike capillary near the cells selected. Gently blowing out the ethanol on the slide, visualize the cells in the droplet of 20% ethanol.
6. Using the capillary tip, gently scrape and separate the cells of interest off the slide. They will usually lift off in clusters, and can be sucked up with the 20% ethanol droplet. Damp remnants can be aggregated and lifted off adhering to the tip (*see* **Note 3**).
7. Transfer to a sterile Eppendorf containing 25 µL of extraction buffer. Repeat the process at various foci of cells in the same section.
8. Add Proteinase K to a concentration of 400 µg/mL.
9. Add a 25 µL mineral oil overlayer.
10. Incubate at 52°C for 2 h and then at 95°C for 45 min. Store at –20°C (long term) or 4°C (short term). As a source of template DNA for PCR, 1–1.5 µL aliquots can be used

3.2. Microsatellite PCR

3.2.1. General Principles

The PCR amplification of microsatellites conforms broadly to the general principles of PCR described by Saiki et al. ***(6)***. Briefly, the reaction mix comprises template DNA, two oligonucleotide primers, the four deoxynucleotides, a buffer with Mg^{2+} ions, and the *Taq* polymerase.

For successful and reproducible microsatellite PCR, additional precautions have to be taken:

1. Template DNA: This may be obtained from a variety of sources (*see* **Subheading 3.1.**). For archival degraded templates, microsatellites smaller than the average size of the nicked template fragments must be chosen. In practice, microsatellites smaller than 180 bp should be readily amplifiable from most sources.
2. Primers: If designing primers from scratch, selecting 18–24 mers with a 35–55% GC composition, matching the %GC of the two primers to within 5% of each other is recommended. Additional precautions against primer complementarity, secondary structure, and *Alu* homology are usually addressed by customized primer design software. Siting the primers as close to the microsatellite as possible enhances amplification efficiencies and rapid fragment separation and

scoring. Often, primer sequence can be accessed from electronic databases Optimal annealing temperatures often need to be empirically determined for the individual laboratory setup. Primer concentrations of 0.1–0 3 μ*M* are generally suitable for microsatellite PCR.

3. dNTPs: Concentrations of between 20 and 100 μ*M* for each nucleotide, lower than the 200 μ*M* used in standard PCR, ensure the higher specificity and fidelity required for microsatellite PCR.
4. PCR Buffers: Buffer conditions need to be individually and empirically optimized for each primer pair, and are discussed in **Subheading 3.2.2.** The use of adjuncts like bovine serum albumin, ammonium sulphate, glycerol, and formamide can be attempted if initial optimization is not fully satisfactory.
5. Thermal Cycling: Thermal cyclers need to have high ramp rates and uniform temperatures across the block, with minimal well to well temperature variation. Thin walled micro tubes (0.2 mL) and small reaction volumes (10 μL) minimize thermal lag between block and sample. The use of hot start PCR is crucial to enhance specificity and sensitivity, especially with low copy number or degraded template DNA.

3.2.2. Optimization of PCR (Nonradioactive)

The amplification conditions for human microsatellites are often found accompanying their sequences (including recommended primers) and locations on electronic databases such as the Genome Database and in published manuscripts. In our experience, some empirical optimization is generally still required because of the variation in the performance of different PCR machines. This is of particular importance when using archival DNA as template, or for quantitative applications such as loss of heterozygosity analyses.

The protocol given below is a useful first (and often only) step in empirically determining optimal conditions for microsatellite PCR. The two major variables assessed are the composition of the 10X PCR buffer (pH and Mg^{2+} ion concentration) and the annealing temperature for the primer pair. Six different 10X buffers are prepared (*see* **Subheading 2.2.**) and tested with a positive template at three different annealing temperatures, in 5°C increments. We use the GeneAmp 9600 system with corresponding 0.2 mL thin walled micro tubes, but the protocol can be easily scaled for use in other formats.

1. In a clean PCR area, set up 21 autoclaved micro tubes on a 9600 base plate. Arrange and label the tubes in three rows of 7 tubes each (six buffers and one PCR blank at three different annealing temperatures).
2. Aliquot 10 μL mineral oil into each tube.
3. Prepare a master mix on ice, sufficient for 24 reactions (21 +3 extra), containing all of the components required for the reaction except the template DNA, 10X reaction buffer, and *Taq*: 20–100 μ*M* each (typically 65 μ*M*) dNTPs, 0.1–0.3 μ*M* each (typically 0.2 μ*M*) primers, and sterile water to a final volume of 7.5 μL.

4. Mix thoroughly by pipeting up and down.
5. Aliquot 7.5 µL into reaction tubes.
6. Add 1 µL of the appropriate 10X PCR buffer (buffers 1–6) into separate tubes, repeat the pattern for the three rows. Use buffer 4 for the blanks.
7. Add 2–10 ng HMW genomic DNA in a volume of 1 µL (or 1 µL of microdissect) Add 1 µL sterile water to the blanks.
8 Denature the genomic DNA by incubating the reactions at 95°C for 5 min in the thermocycler and link this program to "hold" cycle, maintaining the temperature at 85°C for 15 min.
9. During the "hold" step, aliquot 0.05–0.1 U of *Taq* polymerase in a 0.5 µL volume into each tube. Minimize the time taken for this step. The tube should not be out of contact with the hot block for over a few seconds (*see* **Note 5**).
10. Perform between 30 and 40 cycles of PCR. A typical cycle consists of denaturation at 94°C for 12 s, annealing at the determined annealing temperature for 10 s, and extension at 72°C for 10 s (*see* **Note 6**).
11. Incubate at 72°C for 5 min to ensure complete elongation on all single-stranded templates as a final step in the thermal cycling.
12. Store reactions at 4°C.

3.2.3. Radioactive PCR

3.2.3.1. Direct Incorporation of Radionucleotide in PCR (Internal Labeling)

By replacing a dNTP with a radiolabeled analog in the PCR reaction mixture, a radionuclide can be incorporated into the amplified DNA fragments. The proportion of radionuclide incorporation into the synthesized strand can be varied by altering the proportion of the unlabeled nucleotide versus corresponding radiolabeled nucleotide. Equivalent molarities of all four nucleotides must be maintained in the PCR reaction mixture in order to ensure the synthesis of full length products. Protocols are essentially the same as those developed to generate radiolabeled probes by PCR that are discussed elsewhere in this book. In our experience, the incorporation of a radionucleotide into amplified fragments during PCR is not a highly reproducible procedure and leads to difficulties in the resolution and detection of products. This is presumably because of variability in the number of radionuclides incorporated into each fragment, their variable activity, as well as the signal being generated by both product strands during denaturing PAGE autoradiography.

3.2.3.2. Primer 5' Radiolabeling (End Labeling)

This is the preferred method for all microsatellite amplifications in our laboratory. The entire procedure can be performed using the standard equipment found in any molecular biology laboratory; i.e., PCR machine, sequencing gel apparatus, and access to autoradiography facilities. Its advantages are:

1. Fewer cycles are required compared to nonisotopic microsatellite PCR and amplification artefacts are therefore reduced.
2. Very high resolution of the amplified fragments can be achieved using denaturing PAGE.
3. The amplification of the two alleles can be quantitative, and since only one strand of PCR product is labeled, a "cleaner" autoradiograph is obtained on electrophoresis through a denaturing gel.

We use a 10-fold lower concentration of [γ-^{32}P]ATP:pmoles DNA 5' ends than standard protocols *(7)*. The lower incorporation efficiency ensures a cleaner gel and lower reagent costs. If optimal radiolabeling is required, the DNA should be reduced 10-fold and PCR cycles reduced correspondingly.

1. Prepare the kinase reaction mixture by mixing the following components in a sterile microfuge tube on ice, in the order in which they are listed: 6 µL 10X kinase buffer, 300 pmol (3 µL @ 100 pmol/µL, oligonucleotide, 100 µCi [γ-^{32}P]ATP (7000Ci/mmol), 10 U (1 µL @5–15 U/µL) T_4 polynucleotide kinase, and sterile water to a final volume of 60 µL.
2. Incubate at 37°C for 30–45 min (see **Note 7**).
3. Heat inactivate the polynucleotide kinase by incubation at 90°C for 5 min.
4. Pulse reaction tube in a micro centrifuge for 5 s and store on ice.

3.2.3.3. Purification of Labeled Primer

1. Plug the tip of a 1-mL disposable syringe with sterile siliconized glass wool
2. Pack the syringe with Sephadex G-25 which has been pre-swollen in TNES by incubation at 37°C overnight and autoclaved. This is achieved by placing the plugged syringe in a 50-mL Falcon tube through a hole made in the cap using the heated large end of a Pasteur pipet. The slurry is put in the syringe with a sterile 3-mL transfer pipet and the falcon in then spun at 3500*g* for 5 min in a bench-top centrifuge. It can take 3–5 loads of slurry to fill the syringe (*see* **Note 8**).
3. Place a sterile Eppendorf in the Falcon tube below the syringe and apply labeled oligonucleotide to the packed column.
4. Spin column at 3500*g* for 5 min. The labeled oligonucleotide is collected in the Eppendorf tube while [^{32}P]-ATP (and other small molecules such as the Mg^{2+} in the reaction buffer) remains in the Sephadex G-25.
5. Determine the volume of eluate and calculate the concentration of the oligonucleotide.
6. Store the labeled oligonucleotide at –20°C for up to 2 wk (*see* **Note 9**).

3.2.3.4. PCR with Labeled Primer

Reaction conditions are as for non isotopic microsatellite PCR but the reactions are performed with the optimised 10X PCR buffer in a final volume of 10 µL. The number of cycles is reduced to between 20–32 depending on the quality and quantity of template DNA in the reaction and the residual activity of the [^{32}P] ($t^{1/2}$ = 14.3 d).

3.3. Post-PCR Fragment Resolution

The major technical problem in the analysis of microsatellites is the resolution and detection of the amplified DNA. Since polymorphisms between alleles often occur as multiples of 2 bp, high percentage polyacrylamide electrophoresis (PAGE) has to be employed to separate the fragments. The two major options are the use of nondenaturing or denaturing formats. Nondenaturing PAGE has the advantage of being performed in smaller sized formats, making the gel setup and handling easier. Detection of PCR products can be done by intercalating dyes, silver staining, or autoradiography. However, resolution of 2 bp polymorphisms is poorer than with denaturing gel formats. These are more demanding to setup and handle, and usually require radioactive detection of PCR products. Advantages are optimal resolution of fragments and quantitative detection of alleles.

3.3.1. Nondenaturing PAGE

Amplified double-stranded DNA fragments can be separated by nondenaturing PAGE. 12% polyacrylamide gels are suitable for the resolution of DNA fragments that differ in length by 2 bp between 40 and 200 bp. This includes the majority of microsatellites. The microsatellite fragments can be visualized following resolution by exposing the gel to film if a radiolabeled nucleotide has been incorporated into the amplified DNA fragments. The gel may be exposed either wet or after drying. More commonly the amplified DNA is visualized using ethidium bromide or silver staining. The problem with ethidium bromide staining is that it is often not sensitive enough to detect the quantities of DNA amplified. Silver staining has the advantage of greater sensitivity (detects picogram quantities of DNA), however, it is not linearly quantitative and very often high levels of background nonspecific staining are seen. These methods are adequate for qualitative genotyping but are far from ideal when quantitative data is required.

1. Prepare the following gel mixture: 11.8 mL sterile water, 2 mL 10X TBE, 6 mL 40% acrylamide.
2. Siliconize glass gel plates with dimethyldichlorosilane solution and assemble gel pouring apparatus with either 0.5-mm or 0.75-mm spacers.
3. Initiate polymerization by adding 50 µL of TEMED and 150 µL of 10% solution of ammonium persulfate (w/v) to gel mixture and pour gel using a syringe and large bore needle. Insert the comb into the gel before polymerisation is complete (*see* **Note 10**)
3. Assemble polymerised PAGE in vertical tank and wash the wells extensively with the 1X TBE running buffer.
4. Load 10 µL of PCR reaction per lane and 1 µg of pBR322 DNA-*Msp* I digest as molecular weight markers.
5. Gels are run at constant power of 7 W for 3–5 h with cooling (*see* **Note 11**).

3.3.1.1. Ethidium Bromide Staining

1. Separate the gel plates and immerse the plate to which the gel is attached in 1X TBE containing 0.5 μg/mL ethidium bromide for 5 min (*see* **Note 12**).
2. Remove the TBE containing 0.5 μg/mL ethidium bromide and replace with an excess of 1X TBE.
3. Shake on an orbital platform for 10 min.
4. Repeat **steps 2** and **3** twice.
5. Remove gel and visualize resolved fragments using shortwave ultraviolet light.

3.3.1.2. Silver Staining

1. Clean a large petri dish with concentrated nitric acid and then rinse extensively with double distilled water
2. Immerse the gel, that should still be attached to a glass plate, in 200 mL fixative solution.
3. Shake for 20 min and then aspirate off the fix solution. The plate and gel will separate during the shaking. The plate should be removed.
4. Wash in 200 mL of water for 20 min twice.
5. Wash in 50 mL of a 5% solution of reduction moderator for 10 min
6. Wash in 200 mL of water for 20 min twice.
7. Aspirate off all traces of water and add development solution prepared by mixing the following components (provided by the BioRad Silver stain Plus Kit) in the order in which they are listed with vigorous shaking: 17 mL sterile water, 2.5 mL silver complex solution, 2.5 mL reduction moderator solution, 2 5 mL image development solution, and 25 mL developer enhancer.
8. Allow stain to develop for 20 min to 1 h.
9. Stop reaction in 5% glacial acetic acid for 1 h in the dark.
10. Gels can be dried for storage or photographed.

3.3.2. Denaturing PAGE

Microsatellites can be resolved on denaturing sequencing gels and subsequently visualized using film if they are amplified with a radiolabeled primer or with the incorporation of a radionucleotide. Amplification using a kinased primer is preferred to amplification with incorporation of a radiolabelled nucleotide for two reasons: First, if a nucleotide is incorporated, two bands are seen on the gel for each allele since the two single strands from each has a different purine/pyrimidine ratio (and therefore weight). Second, longer microsatellite alleles can be expected to have more of the radiolabeled nucleotide incorporated into them by the polymerase than shorter alleles. Although this is expected to be a linear process, it is a potential source of error that can be avoided for quantitative microsatellite PCR. Using a kinased oligonucleotide ensures that a single molecule of [^{32}P] is incorporated into each microsatellite fragment synthesized during PCR.

Single-stranded DNA fragments that differ in length by 2 bp are well resolved on 6% denaturing urea sequencing gels. These are fixed, dried, and then exposed to film at –70°C using intensifying screens if the film is preflashed. The film must be preflashed to an OD of 0.1–0.2 at 540 nm whenever intensifying screens are used in order to ensure that its response is linear *(8)*.

3 3 2.1. Setting up a Denaturing Polyacrylamide Gel

1. Clean gel plates with hot water and a nonscratch liquid detergent. Wipe dry with soft tissues, followed by ethanol. Then siliconize both the dry, clean plates with dimethyldichlorosilane solution
2. Place mirrored/glass back gel plate raised by about 2 cm horizontally on the bench. Lay slightly damp 0.4-mm thick spacers along the longitudinal edges of the back plate and clamp them in place at the top of the gel Wipe the top of the spacers dry.
3. Prepare the following gel mixture.12 mL Sequagel concentrate, 32 mL Sequagel diluent, and 6 mL Sequagel Buffer
4. Polymerization is initiated by the addition of 35 µL of TEMED and 600 µL of ammonium persulfate solution (10% w/v)
5 Lay the top end of the glass plate on top of the base of the back plate and pour a small amount of the gel mixture in front of the top plate Gradually slide the top plate over the back plate, while pouring more gel solution onto the back plate just ahead of the top plate Capillary action allows the formation of a continuous thin layer of gel between the plates until both plates are in place
6. Insert a sharks-tooth comb into the gel before polymerization is complete
7. Following polymerization, reverse the sharks tooth comb and wash the wells extensively with 1X TBE (*see* **Note 13**).
8 Prerun the gel at 50 W for 30 min before loading the samples in order to obtain even heating.

3.3.2.2. Denaturing Gel Electrophoresis

1. Add an 0.4 vol of running dye to the PCR reactions through the mineral oil
2. Heat samples to 90°C for 3 min in order to denature the amplified DNA and store on ice.
3. Wash the wells extensively with 1X TBE
4. Load 2.5 µL of sample per track on the gel (*see* **Note 14**)
5. Electrophoresis is performed at a constant power of 50 W for 2.5–5 h (*see* **Note 15**)

3.3.2.3. Fixation and Autoradiography

1. Separate the plates and lay the plate with the gel attached to it, gel side up, horizontally on the bench.
2. Pour fixative solution onto the gel and leave for 20 min (*see* **Note 16**).
2. Pour the fix off the gel and transfer the gel onto dry Whatman 3MM paper.
3 Dry gel at 80°C for 0 5–1 h under vacuum

4. Cover the gel in clingfilm and expose at room temperature to autoradiography film without intensifying screens or at –70°C with two intensifying screens to film preflashed to an OD of 0.1–0.2 at 540 nm. The length of exposure is dependent on the intensity of the signal, and is shorter with the use of intensifying screens and preflashed film

3.3.3. Fragment Analysis

For qualitative analysis of microsatellites, visual inspection is sufficient to determine the presence or absence of alleles, and can be performed on PCR products detected with ethidium bromide, silver stains, or radiation. For such applications, the use of nondenaturing PAGE and simple staining methods is often suitable.

For quantitative analysis, we recommend end labeling a primer for microsatellite PCR, resolving the fragments on a denaturing gel and detecting alleles on preflashed film. Subsequent determination of relative allele intensities will then reflect differences in allele copy number in the template. Subjective methods like visual inspection are prone to error, and we recommend the use of further analytical methods like laser densitometry to integrate allele intensities on the autoradiograph. If a dedicated machine is not available, it may be possible to input the image into a PC using a high resolution scanner or CCD camera. This greyscale image can be analyzed with densitometry software (e.g., Quantiscan, Biosoft UK Ltd.) at a fraction of the cost of a densitometer.

3.3.4. Further Considerations

3.3.4.1. Microsatellite PCR Amplification Artefacts

The interpretation of microsatellite PCR, following the resolution of the major DNA fragments amplified from each allele is complicated by the ubiquitously observed "stutter bands" (**Fig. 2**). "Stutter bands" are artefacts of amplification that vary in length around the major fragments by either 1 bp, 2 bp, or multiples thereof. They can be both smaller and larger than the major bands and are produced by "slipped strand mispairing," a process in which the *Taq* polymerase and the DNA strand it is synthesizing slip forward or backward to the next dinucleotide repeat (since the two strands still base pair correctly following such an event, DNA synthesis is not halted). The number of "stutter bands" amplified in a reaction is dependent on the number of amplification cycles performed and the purity of the genomic DNA amplified. Thus, amplification of a microsatellite with a radiolabeled primer results in less "stutter bands" than nonisotopic amplification.

If the polymorphism of a microsatellite is small, then both of the major alleles amplified are overlapped by artefactual "stutter bands." Typically, genetic mapping can still be performed with a small polymorphism (differing by a few bp)

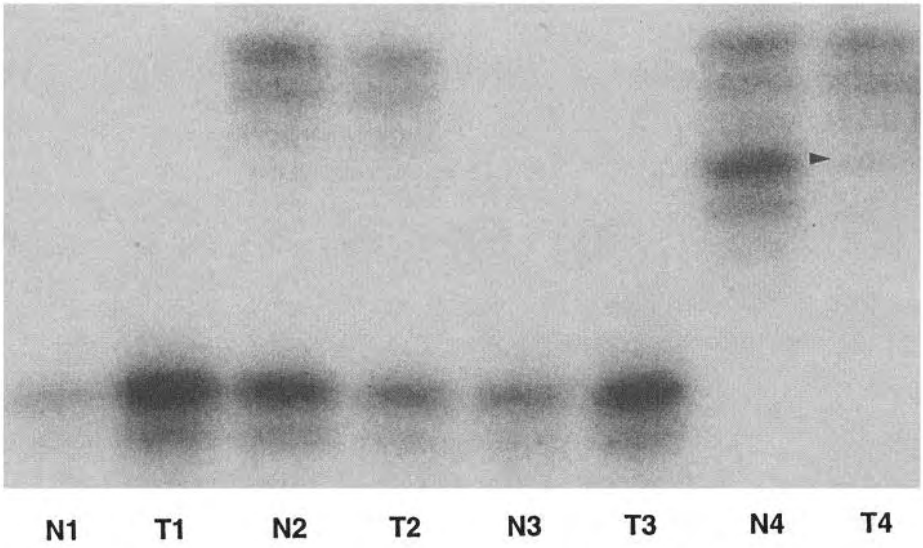

Fig. 2. Autoradiograph of microsatellite alleles amplified from microdissected normal (N) and tumor (T) DNA from four cases. Stutter bands can be seen below the dominant allele bands. Cases 1 and 3 are noninformative (homozygous) at the locus, whereas cases 2 and 4 are informative (heterozygous). In case 4, the tumor DNA shows loss of the smaller allele, indicated by the arrowhead.

if the intensity of the stutter bands is minimal. These extra bands can even be useful as they are diagnostic of microsatellite amplification. To researchers studying allelic imbalance in malignant disease, however, "stutter bands" overlapping the amplified alleles present a major problem. In these instances, it is not clear what bands should be quantitated while assessing allelic imbalance in tumor versus normal tissue. We have observed that microsatellites must be polymorphic by at least 4 bp before they are informative in allelic imbalance experiments. This reduces the number of microsatellites available for study by approximately 40%.

3.3.4.2. Automation of Microsatellite PCR

The genetic mapping of polygenic traits in humans requires hundreds of thousands of genotypes. Futhermore, the identification of linkage to specific chromosome loci predisposing to complex traits requires the use of a genome wide exclusion mapping strategy; i.e., in polygenic diseases, all regions of the genome that are not linked with genetic predisposition must be eliminated as potential predisposition loci, as opposed to a locus causing a monogenic disease that must be identified in all individuals who have the disease. Conven-

tional nonisotopic and autoradiographic protocols for microsatellite PCR require multiple steps and are extremely time consuming; DNA fragments amplified using a radiolabeled primer can take anything from 12–72 h to visualize by autoradiography and the manual interpretation of DNA patterns is complicated. The use of fluorescence technology for genotyping microsatellites, greatly increases the speed of microsatellite PCR and eliminates many of the problems associated with data interpretation. This technology should also greatly assist the study of allelic imbalance in malignant disease.

3.3.4.3. Fluorescent Microsatellite PCR

In order to use fluorescent technology, one of the PCR primers can be labeled at its 5' end with one of four fluorescent phosphoramidites during oligonucleotide synthesis, each of which has a different emission wavelength. The fluorescent dyes are FAM (blue), JOE (green), TAMRA (yellow) and ROX (red) (Applied Biosystems). Alternatively a fluorescently labeled dNTP (R110, blue; R6G, green; TAMRA, yellow) can be incorporated into the microsatellite fragments amplified during PCR reaction (but this is likely to have the same drawbacks as those described for the incorporation of a radionucleotide). The fluorescently labeled PCR products are resolved on polyacrylamide gels and detected when excited to fluoresce by a laser situated at the base of the gel. The fluorescence is detected by a photo multiplier or charged couple device (CCD) camera and quantitated automatically by computer software. These detection devices have a larger response linearity than autoradiographic film and are extremely sensitive; less than 20 cycles are required for microsatellite PCR. Thus, the number and intensity of the "stutter bands" is decreased using fluorescent technology. Coupled with the automatic quantitation of the fluorescence, this makes data interpretation considerably faster. Since the four dyes have different emission wavelength, four PCR reactions can be run in each lane of the polyacrylamide gel at a time. Thus, throughput is greatly enhanced. The reproducibility and sensitivity of fluorescent, versus autoradiographic protocols for microsatellite PCR, has found that fluorescent based technology is at least as accurate as standard radiolabeling protocols ***(9)***. This requires expensive dedicated hardware and software and is a specialized subject in its own right.

4. Notes

1. The plastic Micropestles fit 1.5- and 2.0-mL Eppendorf brand microfuge tubes exactly This tight fit ensures complete tissue homogenization. The Micropestles can be cleaned between samples by incubating in glass coplin jars of concentrated HCl for 10 min, followed by three serial washes in distilled water and a final wash in 70% ethanol.

2. When drawing out the glass pipet in the bunsen flame, it is better not to pull out the capillary straight, in the line of the pipet, but at an angle to the pipet. The downward pointing capillary so produced is easier to use for microdissection.
3. An alternative to the use of a custom made capillary, especially for somewhat larger dissection areas, is to use a narrow gauge needle (G22-G25) attached to a 1-mL insulin syringe as a microdissection tool.
4 The Perkin-Elmer 9600 thermal cycler has a heated lid for oil free operation However, we have noted that condensate collects on the sides of the tubes during oil free cycling. At total reaction volumes of 10 μL, this can significantly alter reactant concentrations during PCR. Mineral oil prevents this occurrence.
5. As an alternative to manual hot start, the use of wax beads (Ampliwax™), *Taq* antibodies (Taqstart™), or heat activated *Taq* polymerase (Ampli*Taq* Gold™) can be considered. For a 10 μL format, we find that Ampli*Taq* Gold is a good alternative, if the added cost is affordable.
6. The optimum annealing temperature will typically be between 3°C and 10°C above the average melting temperature (T_m) of the oligonucleotides calculated using the Wallace rule: $T_m = (4 \times \text{no. of G/C bonds}) + (2 \times \text{no of A/T bonds})$ ***(10)***.
7. Since the kinase reaction is an exchange reaction there is no point in incubating for longer than 30–45 min.
8 Alternatively, it can be quicker to repeatedly compact the slurry with the pipet plunger till the packer slurry reaches the 1 mL mark and then spin the syringe for a final compaction.
9. A rough estimate of the efficiency of the kinase reaction can be achieved by monitoring the counts per second of the solution that elutes from the column and that which remain in it using a beta radiation monitor (Geiger counter). Incorporation sufficient for microsatellite PCR is indicated if equivalent counts are detected in the column and in the solution.
10. The polymerization of acrylamide is an endothermic reaction Therefore, if polymerization is to be accelerated the gel should be placed at higher temperature, and if polymerization is to be slowed, the gel mixture should be cooled on ice prior to the addition of TEMED and ammonium persulfate
11 In 12% nondenaturing PAGE, bromophenol blue migrates with 20-bp DNA fragments and xylene cyanol FF migrates with 70-bp DNA fragments.
12. It is useful to line the inside of the staining container in Saran wrap. The gel can be removed from the container after staining using the cling film if it comes away from the glass plate.
13. It is useful to lightly coat the sharks tooth comb with mineral oil before reversal to minimise leakage of PCR product between wells during loading.
14. A sequencing ladder may be run on the gel as molecular weight markers. We use the M13 phage positive control in the Sequenase Version 2 0 DNA sequencing kit (Amersham 70770).
15. In a 6% sequencing gel, bromophenol blue migrates with 45 bp single-stranded DNA fragments while xylene cyanol FF migrates with DNA fragments of approx 160 bp.

16. Fixation facilitates drying of the sequencing gel and increases the resolution of the signal on film but is not essential.

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