

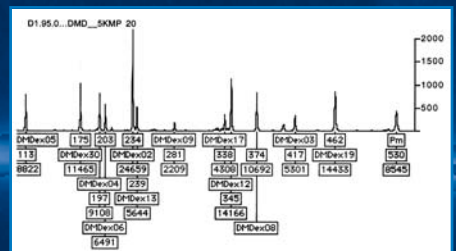
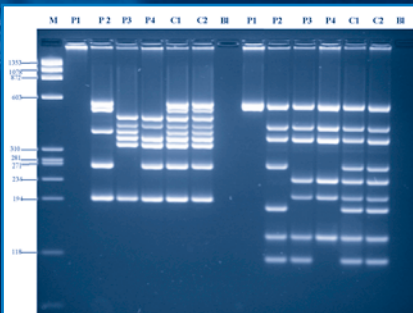
# Molecular Diagnosis of Genetic Diseases

*Second Edition*

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## Optimizing PCR for Clinical Diagnosis

Michael P. Bulman

### 1. Introduction

The polymerase chain reaction (PCR) has rapidly become an essential tool within the diagnostic laboratory. Therefore, it is crucial when setting up a new PCR-based test to ensure that the PCR reaction is carefully designed to be as robust and reliable as possible. Usually, little optimization is required. However, there are some instances when a particular region of DNA proves difficult to amplify by PCR. A number of factors are important to consider when choosing PCR conditions, and these are discussed in this chapter.

### 2. Materials

Analytical-grade reagents should be used at all stages, unless otherwise stated.

#### 2.1. PCR Reaction Buffers

As an alternative to the buffer supplied by the manufacturer, use of either of the following buffers may be beneficial in the PCR.

##### 2.1.1. Buffer "A" 10X

1. 670 mM Tris-HCl (pH 8.0 at 25°C).
2. 166 mM ammonium sulfate.
3. 37 mM magnesium chloride.
4. 67  $\mu$ M ethylenediaminetetraacetic acid (EDTA).
5. 0.85 mg/mL bovine serum albumin (BSA).

Filter-sterilize and store in 1-mL aliquots at  $-20^{\circ}\text{C}$ .

This is a particularly robust PCR buffer, which provides a good yield of product, and is excellent regardless of the quality of the DNA template.

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### 2.1.2. Buffer “R” 20X

1. 1.0 M Tris-HCl (pH 9.0 at 25°C).
2. 400 mM ammonium sulfate.
3. 30 mM magnesium chloride.

Filter-sterilize and store in 1-mL aliquots at  $-20^{\circ}\text{C}$ .

This buffer is useful for templates that are difficult to amplify, such as those with GC-rich tracts, and for longer templates (800 basepairs [bp] + in size).

### 2.2. Deoxynucleotide Triphosphates (dNTPs)

Purchase individual dNTPs as 100 mM stocks (e.g., Roche Diagnostics UK Ltd, Lewes, UK; dNTP set #1969064). Make a 2.5-mM working stock by combining 2.5  $\mu\text{L}$  of each dNTP and adding 90  $\mu\text{L}$  of water. Store dNTPs in small aliquots at  $-20^{\circ}\text{C}$  and avoid multiple freeze-thaw cycles, as dNTPs are prone to degradation (especially for multiplex PCR). The final concentration of each dNTP in the PCR reaction is usually 200–250  $\mu\text{M}$ . To increase the specificity/fidelity of the PCR, decrease the concentration of dNTPs with a corresponding decrease in  $\text{MgCl}_2$  concentration.

### 2.3. Magnesium Chloride (if required)

10X “A” and 20X “R” buffers already contain magnesium at 37 mM and 30 mM respectively (to give a final concentration in the PCR reaction of 3.7 mM and 1.5 mM, respectively). However, for those 10X buffers that do not contain magnesium, this is usually supplied as a 25–50-mM stock and should be used at 1.5–5.0 mM final concentration in the PCR. Mix the magnesium thoroughly prior to addition to the PCR mix.

### 2.4. Oligonucleotide Primers

The final concentration in a PCR reaction should be between 0.1  $\mu\text{M}$  and 0.5  $\mu\text{M}$  of each primer. Primer concentrations that are too high may lead to mispriming in the reaction. Conversely, primer concentrations that are too low may not give good yields of product.

### 2.5. Polymerases

There are many suppliers and varieties of heat-stable *Taq* polymerases on the market. (See <http://www.alkami.com/methods/refpoly.htm> and/or [http://www.neb.com/neb/frame\\_tech.html](http://www.neb.com/neb/frame_tech.html) for information of most of those available.) The most frequently used enzyme is *Taq* polymerase. This enzyme does not have a 3′–5′ exonuclease activity, which has two consequences: it exhibits a non-template addition of usually an adenine base at the 3′ end of the product (*I*); secondly, the lack of the 3′–5′ exonuclease activity means that *Taq* does not correct for the incorporation of mismatched bases—it has no “proof-

reading” activity (2–5). Therefore, for those techniques such as PCR-based site-directed mutagenesis where a reduced error rate is crucial, it is important to use a proof-reading enzyme such as *Pfu* from Stratagene (La Jolla, CA) (6) or *Vent*<sup>TM</sup> polymerase from New England Biolabs (Hitchin, Hertfordshire, UK) (7). For the vast majority of applications, however, *Taq* is perfectly adequate.

## 2.6. Template DNA

Dilute good-quality genomic DNA in TE or deionized water to approx 25–50 ng/μL and use 2.5-μL in a 25-μL reaction.

## 3. Methods

Unfortunately, there is no single set of conditions that can be applied to all PCR amplifications. Factors such as primer sequence, product length, and primer annealing temperature will differ for each assay. For the reliable amplification of a specific target, the optimal conditions for PCR will be found empirically. However, a well-designed PCR reaction should work with little or no optimization necessary.

### 3.1. Design of PCR Primer Pairs

The selection of the correct pair of primer sequences for the PCR reaction may be the most critical parameter for successful PCR. The primer set must hybridize efficiently to the target sequence with as little hybridization as possible to other sequences also present in the sample. Poorly designed primers may result in the synthesis of little or no product as a result of “primer-dimer” formation and/or nonspecific amplification.

### 3.2. Primer Length

In general, oligonucleotides between 20 and 30 bases are sufficiently sequence-specific for complex genomes, provided that the annealing temperature is optimal.

### 3.3. GC Content

Ideally, primer sequences should be designed to have a GC content between 45% and 55%. Stretches of poly C or poly G should be avoided, as these can promote nonspecific annealing. Similarly, stretches of poly A or poly T should also be avoided, as these may open up stretches of the primer-template complex.

### 3.4. Melting Temperature ( $T_m$ )

As a starting point, the annealing temperature for a primer pair is calculated as 5°C below the estimated  $T_m$ . Ideally the primers should closely match each other in their melting temperatures, or amplification efficiency will be reduced and may even lead to the failure of the PCR.

A rough and ready way to calculate  $T_m$  for primers <20 bp is (8):

$$T_m = [4(G+C) + 2(A+T)]^\circ\text{C} \quad (1)$$

To calculate  $T_m$  for primers >20 bp use (9):

$$T_m = 62.3^\circ\text{C} + 0.41^\circ\text{C} (\%G-C) - 500/\text{length} \quad (2)$$

Online  $T_m$  calculators such as that found at <http://alces.med.umn.edu/rawtm.html> are also extremely useful.

### 3.5. General Comments on Primer Design

Alternatively, use a free web tool such as *Primer3* (available at [http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)) or a commercially available program such as Oligo 6 primer analysis software (Molecular Biology Insights Inc., Cascade, CO; <http://www.oligo.net>) to help design primers.

In conclusion, an “ideal” primer will have a 50% GC content with a near random nucleotide composition, and will be 20–25 bp long resulting in a  $T_m$  of 56–62°C. However, primers can only be designed from the available sequence, and sometimes primer design is tricky. Compromise is the key, and it is very unusual to be unable to design a primer pair that will work after some optimization.

### 3.6. Standard PCR Reaction

A standard 25 µl PCR reaction contains the following components:

<u>Component</u>	<u>Volume</u>
10X buffer	2.5 µL
dNTP mix 2.5 mM each	2.5 µL
5 µM primer mix	5.0 µL
Taq polymerase (5 U/µL)	0.05–0.2 µL
DNA template (~25–50 ng/µL)	2.5 µL
Sterile deionized water	to 25 µL

It is important to mix all components thoroughly after thawing prior to assembly of the PCR master mix.

Note: It is essential to include a water “negative control” for each PCR setup, otherwise contamination in the reaction components is not apparent.

### 3.7. PCR Program

#### 3.7.1. Denaturing Temperature

This is normally 94–95°C for 30 s to 1 min per cycle after an initial 3–5 min incubation at 95°C. It is important to keep the denaturation temperature/time to a minimum, since *Taq* polymerase may lose activity in repeated cycling.

### 3.7.2. Annealing Temperature

See **Subheading 3.4.** to estimate the  $T_m$  and anneal at 5°C below this temperature.

### 3.7.3. Elongation Temperature and Time

The elongation temperature is normally 70–72°C, and the elongation time depends on the size of the final size of the PCR product. Generally, 30 s to 2 min is sufficient for most PCR reactions; however, for larger products, a general rule is to extend for 1 min per kilobase of product size.

### 3.7.4. Cycle Number

The standard number of cycles necessary for efficient amplification is 25–40 cycles. Increasing this to >40 cycles does not generally increase the relative amount of PCR product because of the plateau effect, in which the exponential rate of product accumulation in the later stages of PCR is attenuated (**10**).

### 3.7.5. Final Extension

Program one final extension cycle for 7–10 min at 72°C to fill in any incomplete polymerization. Then cool the reaction to 4–18°C.

### 3.7.6. Thermal Cyclers

Many thermal cyclers are available. Some of these are compared at <http://www.biocompare.com/molbio.asp?catid=33>. The author uses the 0.5-mL GeneAmp® PCR System 9700 (Applied Biosystems [ABI], Foster City, CA) and the MJ Research DNA Engine Tetrad™ Thermal Cycler (Genetic Research Instrumentation Ltd, Braintree, Essex, UK).

## 4. Notes

Most manufacturers of PCR reagents and equipment have excellent web sites with useful online guides that can often be downloaded as PDFs (e.g., Qiagen at <http://www.qiagen.com/literature/pcrlit.asp>; ABI at <http://www.appliedbiosystems.com/support/techtools/>; Invitrogen at <http://www.invitrogen.com/content.cfm?pageid=4155&cfid=6398854&cftoken=97182355>).

### 4.1. Hot Start PCR

PCR hot start can be used to increase the reaction sensitivity, reduce non-specific products in the PCR, and increase the PCR yield. The simplest way to set up hot start PCR is to use one of the chemically modified *Taq* polymerases

such as AmpliTaq Gold (ABI). For details, see <http://www.appliedbiosystems.com/products/productdetail.cfm?ID = 104> or Platinum Taq (Invitrogen Ltd., Paisley, UK).

#### 4.2. Enhancers for PCR

It may be beneficial to use one or more additives to increase the yield, specificity, and consistency of the PCR reaction. A variety of such agents are available, including dimethyl sulfoxide (DMSO), dimethyl formamide, betaine (N, N, N-trimethylglycine = [carboxymethyl] trimethylammonium) formamide, 7-deaza-2'-deoxyguanosine (7 deaza GTP), non-ionic detergents (e.g., Triton X-100, Tween 20, and Nonidet P-40), BSA, urea, and glycerol. These additives are believed to lower the  $T_m$  of the target DNA. A helpful discussion of the benefits of the most useful additives can be found at Rob Cruickshank's PCR additive page at <http://taxonomy.zoology.gla.ac.uk/~rcruicks/additives.html>

Two of the most commonly used PCR additives are the organic solvents DMSO and betaine. Both of these are particularly useful for GC-rich templates by acting as helix destabilizers. DMSO is included in the PCR reaction at a final concentration of 5–10% (v/v), although DMSO at 10% and higher has been shown to reduce the activity of *Taq* by up to 50% (**10,11**). Betaine is used at a final concentration of 1 M (from a 5-M stock in water), and is often included in commercial PCR kits as an “unidentified” additive.

Recently, a number of novel potent PCR enhancers have been discovered, and the most effective of these is tetramethylene sulfoxide (**12**). Use of low-mol-wt compounds such as this has been shown to be more beneficial in the amplification of high GC-rich templates than DMSO and betaine.

#### 4.3. PCR Mixture for Difficult to Amplify Templates

<u>Compound</u>	<u>Volume</u>
10X PCR buffer	2.5 $\mu$ L
Modified dNTP mix*	1.0 $\mu$ L
5 $\mu$ M primer mix	5.0 $\mu$ L
1 mM 7-deaza GTP	1.25 $\mu$ L
DMSO	1.25 $\mu$ L
5 M betaine	5.0 $\mu$ L
Amplitaq Gold™	0.25 $\mu$ L
DNA (25 ng/ $\mu$ L)	5.0 $\mu$ L
Sterile deionized water	To 25 $\mu$ L

\*Modified dNTP mixture contains: 5  $\mu$ L each of dTTP, dCTP, and dATP plus 3.8  $\mu$ L of dGTP made up to 100  $\mu$ L with water (100-mM stocks of each dNTP).



Cycling conditions:

1. 95°C 12 min 1 cycle
2. 95°C 1 min
3. X°C\* 1 min } 40 cycles
4. 72°C 2 min
5. 72°C 10 min 1 cycle

\*Annealing temperature varies depending on the primer set.

#### 4.4. Multiplex PCR

This is a demanding technique often used to amplify several PCR products in a single reaction. Extensive optimization is often required to produce a robust and reliable PCR reaction as with multiple primer pairs in a single-tube reaction, which increases the likelihood of primer-dimer and other nonspecific products that may interfere with the amplification of the products required. An extensive troubleshooting guide for multiplex PCR can be found at <http://www.info.med.yale.edu/genetics/ward/tavi/Trblesht.html>.

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## Current and Emerging Techniques for Diagnostic Mutation Detection

*An Overview of Methods for Mutation Detection*

**Claire F. Taylor and Graham R. Taylor**

### 1. Mutation Detection: An Introduction

This chapter provides a broad overview of the range of mutation detection techniques that are now available.

For the purposes of this chapter, a mutation can be defined as a sequence change in a test sample compared with the sequence of a reference standard. This definition implies nothing about the phenotypic consequences (e.g., pathogenicity) of a mutation. A polymorphism may be defined as a mutation that occurs in a substantial proportion ( $>1\%$ ) of a population and is tacitly assumed to be non-pathogenic, although the true pathogenicity may be unknown. A polymorphism has also been defined as a Mendelian trait that exists in the population, with the frequency of the more rare of the two alleles greater than 1–2% (*I*). If we accept that DNA sequence is a Mendelian trait, then the two definitions of polymorphism are the same.

The detection of a single base change in the human genome requires a signal:background ratio of  $1:6 \times 10^9$ —a formidable task. To achieve such selectivity in the field of electronics would require amplification and noise reduction, and it is no surprise that analogous processes are found in molecular genetics—for example, amplification by the polymerase chain reaction (PCR) and noise reduction by the stringent annealing of probes and primers.

Mutation detection techniques can be divided into techniques that test for known mutations (genotyping) and those that scan for any mutation in a par-

ticular target region (mutation scanning). Broader aspects of mutation detection include identification of gene dosage alterations, gross re-arrangements, and methylation. There are several well-known genotyping and scanning methods in routine diagnostic use. Many of these are covered in detail in this volume and elsewhere (1,2). This chapter focuses primarily on recent modifications, development, and evaluation of these techniques.

The primary considerations in any approach to mutation detection are sensitivity (the proportion of mutations that can be detected) and specificity (the proportion of false-positives). The cost per genotype and throughput are also important factors in service delivery. It is often difficult to evaluate these features accurately from the published scientific literature—presumably, one of the reasons why the Human Genome Organisation Mutation Detection training courses (<http://www.leeds.ac.uk/cmgs/leedsdna/science/hugo/index.html>) and workshops (<http://www.mutations2001.bled.si/>) have proven so popular.

## 2. Genotyping

Because sequence changes can abolish or create cleavage sites for the wide range of commercially available restriction endonucleases (REs), RE polymorphisms were the first tools used for genetic mapping and diagnosis, in combination with Southern blotting of genomic DNA (3,4). Although there are still some applications—for example, mapping large deletions or rearrangements, for which Southern blotting is the best method—the polymerase chain reaction (PCR) is now the method of choice for routine genotyping (5).

### 2.1. Genotype Analysis Using the PCR

The analysis of restriction fragment length polymorphism (RFLP) is now primarily of historical interest as a first choice for genotyping, although it is a robust method. Amplicons are generated to flank a polymorphic RE site and subjected to digestion, and the presence or absence of the site can be determined by agarose gel electrophoresis of the digested amplicon and visualization using ethidium bromide staining and ultraviolet (UV) illumination. Artificial restriction sites can be produced by incorporating modifications into one of the amplimers to increase the range of polymorphisms that can be examined. The requirement to hold stocks of a range of different REs, and two-step genotyping process (amplification followed by digestion) does not lend itself to either rapid or high-throughput genotyping. Gains in throughput can be achieved using high-density gels such as the microtiter array diagonal gel electrophoresis (MADGE) format (6).

## **2.2. Genotyping for Linkage Analysis**

### *2.2.1. Microsatellite Analysis*

Linkage mapping has been accelerated by the description of microsatellites (7). Microsatellite repeats are mono-, di-, tri-, or tetranucleotide repeats that display polymorphism with respect to the length of the repeat. The origin of this length polymorphism is believed to be “strand slippage” during replication. One strand may form a short hairpin and produce a copy of different length in the daughter strand. The PCR gained widespread usage when microsatellites were first described, and they were ideally suited for analysis by designing PCR primers (amplimers) that bind to unique sequences flanking the microsatellite repeat motif. Amplicons must be sized to within the resolution of the repeat motif to provide a genotype. This requires sizing fragments of approx 100–300 base-pairs in length with an accuracy of  $\pm 1$  basepair. The most accurate way to do this is to use some form of automated fragment analysis equipment such as the commercially available automated DNA sequencers. Using capillary arrays and multiple sample loading, genotyping throughputs of up to 500 per hour can be achieved, a total far beyond the current requirements of diagnostic laboratories.

### *2.2.2. SNPs*

Recently, interest has returned to single-nucleotide polymorphisms (SNPs), of which RE polymorphisms are a subset. SNPs are di-allelic (although in principle there is no reason why a particular base could not be substituted by more than one alternative), and thus less informative individually than microsatellites, but far more abundant (8). The human genome may contain millions of SNPs, yet they are probably more abundant in noncoding regions of the genome. Several efforts are now underway to produce genomic SNP maps (9). Genotyping will then aim to type sets of SNPs, or possibly SNP haplotypes, since it is now becoming clear that recombination preserves blocks of haplotypes (linkage disequilibrium) over substantial physical distances (10). The main appeal of SNPs is the prospect of high-throughput automated analysis using array or “chip” technology (11); however, a variety of generic mutation detection techniques can be adapted for SNP detection.

### *2.2.3. Amplification Refractory Mutation System (ARMS)*

ARMS is a modification of conventional PCR in which one of the amplimers is designed to have the polymorphic base in the template at its 3' position (12). *Taq* polymerase is unable to extend from a mismatched base, and thus the generation of a PCR product occurs only if the 3' base in the primer matches the template. The technique can be multiplexed to type up to 20 SNPs simultane-

ously. In practice, an additional mismatch at the 3' minus 3 nucleotide position is required to destabilize primer binding for a stronger assay. A weakness of the standard ARMS approach is that two tubes (wild-type and mutant) are required for a full genotype. However, by modifying the primer length (or fluorescent label if using fluorescent analysis), it is possible to generate different products from each allele. ARMS is a low-cost approach that can use standard laboratory equipment. Higher throughput can be achieved by using closed-tube assay systems or adaptation of high-throughput gel formats such as the MADGE system (13).

#### 2.2.4. Minisequencing

Minisequencing, also referred to as single-nucleotide primer extension (14) and genetic bit analysis (15), determines the base immediately 3' to a primer by extending the primer by one base only (16). Base extension can be monitored by gel electrophoresis (17), and commercial kits are available to run these assays on DNA sequencers—for example, “SnapShot” from Applied Biosystems (ABI). As with most genotyping assays, if the variant is present as a minority species (for example, in a tumor or a germinal mosaic), the reliability of the assay declines, although increased sensitivity of detection by pretreatment of a mixed population containing H-ras codon 61 mutants has been reported (18) using the MutEx assay (19). High-throughput and solid-phase adaptations have also been described (20). Readily adaptable to microtiter (21), high-performance liquid chromatography (HPLC) (22), and array (23) and mass spectroscopy (24) formats, it has considerable potential as a high-throughput system. Although the original report (16) described detection from genomic DNA without amplification, all subsequent reports have used PCR amplification to prepare primer extension templates. In highly parallel systems, the ability to amplify templates becomes rate-limiting. Minisequencing is thus a flexible method that can operate using fairly basic equipment, or can be adapted to highly automated systems.

#### 2.2.5. TaqMan and Molecular Beacons

TaqMan is a closed-system assay that can be adapted for gene dosage as well as genotyping. Single-nucleotide differences are detected in PCR products by the sequence-specific hybridization of a probe that contains both a fluorochrome and a quencher. When hybridized, the quencher molecule is cleaved, and the bound fluorochrome can be detected by a fluorescence assay. Since it is possible to have different colored fluorochromes, the probes can be differentially labeled, allowing both alleles of an SNP to be typed in the same tube (25). Molecular beacons are hairpin-shaped structures that contain a fluorochrome and a quencher on the 5' and 3' ends. In free solution, the fluorochrome is

quenched, but upon hybridization to a specific target the hairpin opens and the molecule becomes fluorescent. These molecules can be used in a closed system for allelic discrimination of PCR products (26). Both assays can be read in real-time or end-point formats, using fluorescent plate or tube readers. The “Scorpion” assay is an interesting development that combines an amplification primer and beacon-like detection component in the same molecule to enable real-time genotyping (27). The LightCycler system (Idaho Technology, Idaho Falls, ID) uses fluorescence resonance energy transfer (FRET) to perform real-time PCR and genotyping using two oligonucleotides, one carrying an energy acceptor and the other an emitter. The oligonucleotides hybridize in tandem on the template. The first dye (fluorescein) is excited by the LightCycler’s LED (Light Emitting Diode) filtered light source, and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the emitted energy excites the LC Red 640 attached to the second hybridization probe that subsequently emits red fluorescent light at an even longer wavelength. The LightCycler is set to detect the longer wavelength (640 nm) light. Energy transfer is highly dependent on the spacing between the two dye molecules. Only when the molecules are in close proximity (a distance between 1 and 5 nucleotides) is the energy transferred at high efficiency, and fluorescence is proportional to the amount of bound primers.

Once suitable oligonucleotides are designed, the genotyping of a sample is straightforward. The instrument is programmed to amplify the DNA and to perform a melting curve analysis. A perfect match has a higher melting temperature than a mismatch. In this way, the LightCycler directly genotypes a sample after amplification with no additional handling. With dual-color detection, it is possible to simultaneously genotype two different mutations in one PCR run. Although the LightCycler uses a rather idiosyncratic arrangement of sealed glass capillaries, other closed-system plate readers for 96- or 384-well plates and automated plate loading are commercially available. The choice of system will probably depend to a large extent on the cost of consumables.

### 2.2.6. Ligation

The specificity of DNA ligase for perfectly matched double-stranded DNA, particularly thermostable ligase (28,29), has been exploited as a genotyping tool for the ligase chain reaction and the ligase amplification reaction (30). In genetic testing, ligase reactions have been more widely used to genotype PCR products rather than to perform the amplification reaction directly—for example, in the development of an assay to genotype 31 pathogenic variants in the cystic fibrosis gene *ABCC7* (31). Two sets of oligonucleotide probes can be ligated only if they are hybridized to a perfectly matched template, the oligonucleotide ligation assay (OLA). This has been adapted to produce a dual-color

microtiter readout (32) and gel-based systems (33) in which the ligation products are distinguished by fragment color and mobility, enabling automated genotype readout. Ligation systems can also be modified to perform microsatellite genotyping (34). This adaptation has the potential to be developed into an array-based system for microsatellite analysis. Ligation has also been adapted to seal nicked circular probes producing “padlock probes” that can be then amplified by rolling circle replication (35–37).

### 2.2.7. Pyrosequencing

Pyrosequencing is a non-electrophoretic real-time DNA sequencing method that uses a unique approach to read small runs of bases (38). The luciferase-luciferin light release is a detection signal for nucleotide incorporation into target DNA. This method can be adapted for automated high-throughput operation, and has the advantage of typing bases that flank the SNP to confirm that the correct target is being analyzed (39). Pyrosequencing of the human *p53* gene using a nested multiplex PCR method for amplification of exons 5–8 has been described, reporting accurate detection of *p53* mutations and allele distribution (40). If the current length of sequence limitation can be overcome, pyrosequencing has considerable potential as a highly automatable sequencing tool.

### 2.2.8. Invader

Invader technology uses a Flap Endonuclease (FEN) for allele discrimination and a universal FRET reporter system. A study by Mein et al. (41) genotyped three hundred and eighty-four individuals across a panel of 36 SNPs and one insertion-deletion (indel) polymorphism with Invader assays using a PCR product as a template. The average failure rate of 2.3% was mainly associated with PCR failure, and the typing was 99.2% accurate when compared with genotypes that were generated with established techniques. Semi-automated data interpretation allows the generation of approx 25,000 genotypes per person per week, 10-fold greater than gel-based SNP typing and microsatellite typing. Using an “Invader squared” method, Factor V Leiden genotyping has been achieved on genomic DNA samples without prior amplification (42), although most assays in routine use now rely on the PCR to generate templates for genotyping.

### 2.2.9. Hybridization

Allele-specific hybridization (ASH) was one of the early methods of genotyping (43), originally using genomic DNA as template, later with PCR-amplified DNA. By carefully controlling the stringency of hybridization, 18- to 22-mer probes can discriminate between single base substitutions of target. This technique is still used, and forms the basis of some commercial test kits for cystic fibrosis (44) and Human Leukocyte Antigen (HLA) typing (45).



Real-time hybridization analysis (dynamic allele-specific hybridization [DASH]) makes the assay more robust, since the denaturation of probe and target can be monitored over a range of temperatures (46). Hybridization can also be monitored by surface plasmon resonance, enabling optical biosensors to perform automated genotyping (47,48). Using this procedure (49,50), it was possible to perform real-time monitoring of hybridization between target single-stranded PCR products, enabling a one-step, non-radioactive protocol to perform cystic fibrosis diagnosis.

### 2.2.10. Arrays

The idea of using arrays for high-throughput genotyping has been in existence for many years. Early arrays were two-dimensional spots of DNA targets on nylon or nitrocellulose membranes, and the method of detection was ASH (51). This method still has value, and recent improvements in the oligonucleotide-binding capacity of membranes (52) could extend this further. However DNA arrays typically refer to glass, plastic, or silicon supports with either oligonucleotide or cloned DNA attached by adhesion or covalent linkage. Arrays that are mechanically deposited onto a glass microscope slide have feature sizes of approx 200 microns, and are scanned at 5–20-micron resolution. Such arrays can carry 10–15,000 features. Affymetrix manufacture high-density arrays by a proprietary photochemical oligonucleotide synthesis method that can result in a small (10- $\mu$ ) feature size, enabling a large number of 20–24-base oligonucleotide probes to be packed into a small area (53). Although these arrays have had the most success in gene-expression studies, they have not yet produced the anticipated breakthrough in DNA sequencing (or “resequencing”) (54) or mutation scanning, although their use has been reported in ABCC7 (55), mitochondrial (56), and BRCA1 (57) mutation detection. The reason for the limited use of the Affymetrix system for mutation detection thus far lies in its limited sensitivity. Di-deoxy sequencing of the *p53* gene in 100 primary human lung cancers by cycle sequencing was compared with sequence analysis by using the *p53* GeneChip assay (58). The GeneChip assay detected 46 of 52 missense mutations (88%), but 0 of 5 frameshift mutations. The specificity of direct sequencing and of the *p53* GeneChip assay in detecting *p53* mutations were 100% and 98%, respectively. Although more mutations were detected in *p53* by manual sequencing than by use of a *p53* gene chip, direct sequencing and the *p53* GeneChip were not infallible at *p53* mutation detection. In another study (59), reported a 92% sensitivity for the detection of *p53* mutations in a series of 108 ovarian tumors, less than might be expected from a current mutation scanning tool such as denaturing high-performance liquid chromatography (DHPLC). Hybridization may not be the best way to exploit arrayed DNA for mutation detection. Several recent studies have indicated that the use of

primer extension (15,23) or ligation (60) can improve the specificity of mutation detection on arrays. With mechanically prepared arrays, this is not difficult to set up, as the oligonucleotide can be arrayed with the 3' end (the substrate for primer extension) free, and the 5' end anchored. However, in light-directed oligonucleotide synthesis, the 3' end of the probe is anchored to the solid support. Although this is not a problem for ligation reactions, it does mean that direct primer extensions for the arrayed oligonucleotide are not possible. This problem can be circumvented by conducting the primer extension reaction in solution and then capturing the reaction products by means of 5' tags on the substrate with complementary tags on the array (61–63). “Zip-code addressable” arrays provide a generic solution for genotyping, as primer sets can be custom-designed to work on standard chips. The same design principle can be applied to “liquid-phase arrays,” which are latex microbeads that can be sorted using a fluorescence-activated cell sorter (FACS). By addressing each bead with a different tag, up to 96 primer extension reactions can be monitored in a single tube (64,65). The same principle can also be applied to provide templates for ultra-rapid mass-spectroscopic genotyping, which is likely to be the method of choice for ultra high-throughput genotyping (24). Here, primer extension products are simply weighed to determine the nucleotide added. Commercial systems are available that include primer design software, sets of validated SNPs, and high-throughput genotype analysis software.

### 3. Dosage

Although methods for the detection of point mutations and small insertions or deletions in DNA are well-established, the detection of larger (>100 bp) genomic duplications or deletions can be more difficult. Most mutation scanning methods use PCR as a first step, but the subsequent analyses are usually qualitative rather than quantitative. Gene dosage methods based on PCR must be absolutely quantitative (i.e., they should report molar quantities of starting material) or semi-quantitative (i.e., they should report gene dosage relative to an internal standard). Without some method of quantitation, heterozygous deletions may be overlooked, and may therefore not be fully evaluated. Gene dosage methods can provide the additional benefit of reporting allele drop-out in the PCR.

Large genomic duplications and deletions have been recognized as pathogenic mutations for many years—for example in alpha-thalassemia (66,67), Duchenne and Becker Muscular Dystrophies (68) and more recently in familial breast cancer (69), and hereditary non-polyposis colorectal cancer (HNPCC) (70,71). Based on the May 2000 Human Gene Mutation Database, deletions and duplications represented 5.5% of reported mutations (72). Because many muta-

**Table 1**  
**Methods Used to Study Dosage**

Method	Resolution (bases)	Comments
Metaphase spread	Chromosomal	Conventional cytogenetic staining
CGH	$5 \times 10^6$	Metaphase spread is used as a probe for test and control differential hybridization (84)
FISH	$5 \times 10^4$	Modifications (e.g., Fibre FISH) to improve resolution (85)
Array CGH	$5 \times 10^4$	Uses arrayed BACs instead of chromosome spreads (86)
MAPH	$1 \times 10^2$	Hybridizes probes to genomic DNA, then amplifies the probes (76)
Microsatellite PCR	Varies	Relies on informative microsatellites being in the region of deletion. Tetranucleotide repeat microsatellites widely used as a rapid aneuploidy detection method (87)
Differential PCR	$1 \times 10^2$	Requires careful control of starting DNA concentration and quality. Gives relative concentrations; thus is semi-quantitative (88)
Competitive PCR	$1 \times 10^2$	Extremely accurate, provided the competitor is accurately dispensed. Gives molar quantities; thus is absolutely quantitative (89)
Real-time PCR	$1 \times 10^2$	More expensive to set up; various detection methods available, including SYBR Green or fluorescent probes in TaqMan or Beacon format (90)
Long-PCR	$1 \times 10^2$	Likely to be more effective in detecting intragenic deletions rather than duplications, and can be used to sequence across the deleted region to establish the precise nature of the mutation (91)

tion scans have not included searches for deletions, it seems likely that these figures are an underestimate. Estimates of gene dosage have typically been based on comparisons with a reference standard; absolute (e.g., molar) quantitation has been reported by the inclusion of known quantities of PCR competitor. Other approaches—including the study of junction fragments or microsatellite inheritance, and more recently, long accurate PCR (73), fluo-

rescence *in situ* hybridization (FISH) (74,75), multiplex amplifiable probe hybridization (MAPH) (76), comparative genomic hybridization (CGH) (77–79), and array-CGH (80)—have also been employed. In some cases, knowledge of the gene (or exon) dosage may not be sufficient to establish the pathogenic consequences of a genotype. For example, in spinal muscular atrophy, in which gene duplications and unstable regions of the genome can complicate the issue (81). Although reciprocal translocations would escape detection by simple dosage techniques, robust low-cost dosage methods may find utility in rapid screening for supernumerary chromosomes (82,83).

Techniques for detecting gene-dosage alterations can be broadly divided into three types: cytogenetic, solid-phase hybridization, or PCR amplification.

#### **4. Methods for Studying DNA Methylation**

In the human genome, DNA methylation is found in the form of 5-methyl cytosine, located almost exclusively within CpG dinucleotides (for a recent review, *see* 92). Perturbations of the normal pattern of methylation are associated with disorders of imprinting and X-chromosome inactivation and also with oncogenesis, and can be considered to be epigenetic mutations.

A number of methods for the study of the pattern of cytosine methylation at specific loci have been described (93,94), all depending on one of three mechanisms to discriminate between methylated and unmethylated cytosines:

- differential cleavage by methylation-sensitive restriction enzymes
- differential cleavage by chemicals
- differential reactivity with sodium bisulphite

##### **4.1. Differential Cleavage by Methylation-Sensitive Restriction Enzymes**

Restriction endonucleases that are unable to cleave DNA when their restriction sites contain 5-methyl cytosine have long been recognized as a tool for the study of cytosine methylation (95). Assays that utilize methylation-dependent restriction enzymes are a more recent advance. Digestion and thus methylation are monitored either by Southern blot or by PCR using primers flanking the restriction site (96,97). These methods are relatively simple and widely used, despite a number of drawbacks that include the confinement of analysis to cytosine residues within restriction sites and the possibility of misleading results as a result of partial digestion or PCR failure.

##### **4.2. Differential Cleavage by Chemicals**

In the Maxam–Gilbert sequencing protocol, hydrazine is used to cleave DNA at cytosine and thymine residues (98). 5-methyl cytosine is resistant to

hydrazine cleavage, and appears as a gap on a Maxam-Gilbert genomic sequencing ladder (99). The original protocol was time-consuming and required large quantities of DNA; later developments such as ligation-mediated PCR (100) addressed a number of these problems. Despite these improvements, the presence of 5-methyl cytosine still must be inferred from the absence of a band, although a protocol allowing the positive display of methylated residues using permanganate has been described (101).

### 4.3. Differential Reactivity with Sodium Bisulfite

Upon reaction with bisulfite, cytosine is deaminated to uracil, whereas 5-methyl cytosine is not reactive. During a subsequent PCR, uracil residues are amplified as thymine, and 5-methyl cytosine is amplified as cytosine. This sequence conversion provides positive identification of methylated residues in the starting sample (102). Direct sequencing of PCR products yields a strand-specific average of the methylation pattern in the starting population of molecules. For information about the methylation pattern of individual molecules, cloning of the PCR products prior to sequencing is required.

Bisulfite modification can lead to the methylation-dependent creation of novel restriction sites or retention of existing sites. PCR followed by restriction digestion provides a rapid method for screening specific CpG sites, which does not rely on an absence of cleavage to detect methylation and can also be used as a quantitative assay (103). Quantification of the level of methylation at specific CpG sites can also be done by a single-nucleotide primer extension assay (MS-SnuPE) (104).

Methylation-specific PCR (MSP) (105) uses PCR primers designed to amplify bisulfite-modified DNA, which can differentiate between methylated and unmethylated DNA. MSP is extremely sensitive, and can detect the presence of methylated DNA at levels as low as 1/1000 (105). A single-tube method for the detection of methylation at 15q11-q13 for the diagnosis of Prader-Willi syndrome and Angelman syndrome (106) is widely used in diagnostic laboratories. A real-time methylation-sensitive PCR has been described, which can be used to quantify methylation (107).

## 5. Mutation Scanning Methods

Mutation scanning is the search for novel sequence variants within a defined DNA fragment. Numerous methods that exploit different physical, chemical, and biological consequences of DNA sequence variation have been developed to facilitate mutation scanning. The ideal mutation scanning method has been characterized as one that would screen kilobase lengths of DNA with 100% sensitivity and specificity, and would completely define the mutation (108). It would be a simple, single-step, non-electrophoretic protocol with high through-

put and low cost, requiring no complex equipment and no harmful reagents. Cost and data-analysis time continue to be major barriers to meeting the demand for genetic testing, and no current method satisfies all of these criteria.

Most scanning methods do not identify the precise nature of the change to the DNA sequence, although some indicate the location of the mutation within the fragment analyzed. Consequently, the majority of methods are used as a first-round screen to identify those samples that contain mutations, and these samples are subsequently sequenced to define the mutations.

Several factors influence the choice of scanning method:

### **5.1. Mutation Detection Sensitivity**

In the clinical diagnostic setting, sensitivity should be as close to 100% as reasonably practicable. Mutation scanning for other purposes such as candidate gene analysis may be able to tolerate a trade-off between a reduction in sensitivity and an increase in throughput. In practice, it is unlikely that any single technique will detect 100% of mutations. An awareness of the limitations of the technique selected is essential. Factors that influence sensitivity include fragment resolution, reactivity of any enzymes or chemicals used, and template features such as sequence (e.g., G + C content), length, and secondary structure. The measurement of sensitivity is empirical: the literature is replete with examples of non-blinded studies or studies using small series, from which it is difficult to draw general conclusions about assay performance.

In a prescreening method, low specificity (large numbers of false-positives) may generate excessive downstream analysis and reduce the advantage of prescreening. Some regions of interest may be highly polymorphic, and generate many samples that require further analysis. Although there have been claims that common polymorphisms generate “characteristic” mobility shifts—for example, in DMPLC HPLC analysis—these claims should be treated with caution in a diagnostic setting.

### **5.2. Suitability for Proposed Sample Type**

Current diagnostic practice is largely restricted to genomic DNA samples extracted from peripheral blood lymphocytes. Future developments are likely to include increasing analysis of DNA extracted from tumor samples, which presents a number of problems that are not encountered when studying germline DNA. In germline samples, mutations can be present at 0% (homozygous or hemizygous wild-type), 50% (heterozygous) or 100% (homozygous or hemizygous mutation) of the total DNA, depending on zygosity, unless mosaicism is present. In tumor samples, the mutation can be present at any proportion of the total DNA because of factors that include loss of heterozygosity, contami-

nation of the tumor with surrounding wild-type material, and variable proportions of mutant cells in the tumor. Some methods such as DHPLC are better able to detect mutations that are present as a minor fraction in the sample (*109*). Many methods are dependent on the generation of heteroduplex DNA for the detection of mutations: depending on whether the expected mutations are likely to be homozygous, hemizygous, or heterozygous it may or may not be necessary to add 50% wild-type DNA to the samples.

### **5.3. Suitability for Predicted Mutation Type**

Some of the methods described here have limitations on the types of mutations they can detect. For instance, DHPLC cannot reliably detect homozygous mutations; heteroduplex analysis (HA) detects insertions/deletions with higher efficiency than substitutions, and the protein truncation test (PTT) detects only polypeptide-chain-terminating mutations.

When the nature of mutation is unknown, a detection method that is unbiased toward any type of mutation should be used. For conditions/genes in which a single type of mutation predominates, it may be more appropriate to select a method designed to detect only that type of mutation.

### **5.4. Features of the DNA Sequence Analyzed**

Knowledge of the presence of common polymorphisms in the fragment to be analyzed may also affect the choice of method. With the exception of the scanning methods that unambiguously identify the mutation present, in most cases the available information will only indicate that a mutation is present or absent. Some methods—for instance, DHPLC and fluorescent single strand conformation polymorphism (FSSCP)—may produce a mutation profile, which, at least superficially, appears characteristic for the mutation (*110,111*), but there is evidence to suggest that this may be unreliable (*112,113*). Thus, it is usually necessary to sequence all samples showing a change from the wild-type pattern. Thus, in the presence of a common polymorphism, a large proportion of samples may require analysis by both a scanning method and DNA sequencing and in these cases DNA sequencing alone may be a more suitable choice.

### **5.5. Health and Safety Considerations**

Both legislation and good practice require that, as much as reasonably practicable, when alternative techniques are available, the safer option should be chosen. Non-radioactive detection methods are thus preferable to radioactive detection, and methods that avoid the use of toxic chemicals are preferable to those methods that are dependent on the use of toxic chemicals.



### **5.6. Expected Requirements for Sample Throughput**

As the expected throughput increases, it becomes necessary to increase automation, decrease analysis time and complexity, decrease the number of manipulations, and increase the level of multiplexing (reviewed in *114*).

### **5.7. Capital Equipment Costs and Ongoing Running Costs**

DHPLC, microarrays, and any technique that requires fluorescent labeling and detection requires a significant investment in equipment before the technique can be established in a laboratory.

### **5.8. Requirement for Post-PCR Manipulation**

It is usually advantageous to minimize the number of post-PCR manipulations for several reasons. The more stages involved in an assay, the greater the likelihood for operator error. Complex techniques are usually low-throughput and less amenable to automation. Additionally, a requirement for post-PCR reactions will result in an increase in the cost per genotype.

Although there are many different mutation scanning methods, most can be fitted into one of four categories: physical methods (which depend upon the presence of a mutation changing the physical properties of the DNA molecule), cleavage methods (which identify the presence of a mutation by the differential cleavage of wild-type and mutant DNA), and methods that detect the consequence of mutation in a protein molecule or a functional assay. Finally, direct sequencing can itself be used to detect mutations.

## **6. Physical Methods**

For physical methods, the practical result of sequence variation is a differential physical property of wild-type vs mutant DNA—for example, gel mobility or homoduplex stability. Although physical methods typically require little post-PCR manipulation and can be performed in a low-technology format using routine laboratory equipment, throughput and sensitivity have been enhanced by the utilization of fluorescent labeling and automated detection.

### **6.1. Single-Strand Conformation Polymorphism (SSCP)**

Single-stranded DNA in non-denaturing solution folds in a sequence-specific manner. A change in the DNA sequence causes a change in the folded structure, which in turn alters the mobility of the conformer on a non-denaturing gel (*115*). The sensitivity reported for SSCP ranges between 35% and 100%, although the majority of studies detected more than 80% of mutations. Multiple conditions of analysis can be used to increase the sensitivity (*116,117*). One major limitation for SSCP is fragment size: a study by Sheffield et al. (*118*)

reported that sensitivity varied dramatically with fragment size, and that the optimum size was as little as approx 150 bp. Three hundred bp is generally regarded as the upper limit for fragment size (119). Utilization of fluorescence and capillary electrophoresis (CE) technology has resulted in higher sensitivities in blinded trials, and may allow high-sensitivity detection in larger fragments (120–122).

Dideoxy-fingerprinting (ddF) is an interesting variant of the SSCP method, in which chain-terminated products are analyzed by SSCP, resulting in increased sensitivity but a rather complex image to analyze (123). Very high sensitivity has been reported using ddF on a high throughput CE system (124).

### **6.2. Heteroduplex Analysis (HA) and Conformation-Sensitive Gel Electrophoresis (CSGE)**

On electrophoresis in a non-denaturing gel, heteroduplexes have retarded mobility compared to homoduplexes (125). The technique was first described for insertion/deletion mutations, but can also be applied to single-base mismatches (126). HA has been successfully applied to fragments of >1 kb in size, although evidence suggests that mutation detection efficiency may be reduced in larger fragments (127). Like SSCP, HA is a very simple technique, requiring no DNA labeling or specialized equipment, and the two techniques can be run together on a single gel (128).

Conformation-sensitive gel electrophoresis (CSGE) is a variant of the HA method, employing mildly denaturing gel conditions (129). For fragments in the size range of 200–800 bp, sensitivity of 88% has been detected, and a reduction in the maximum size of the fragment has been associated with an increase in the detection rate to 100% (130). Mutations within 50 bp of the end of a fragment are not detected, presumably because the distortion of the duplex is not great enough to generate a significant mobility shift (129). Recent developments in CSGE include the application of fluorescent labeling and detection (131,132) and capillary electrophoresis (133).

### **6.3. Denaturing Gradient Gel Electrophoresis (DGGE)**

In DGGE (134), duplex DNA is electrophoresed through a gradient of increasing denaturant concentration. At a characteristic point in this gradient, the duplex will become partially denatured, and electrophoretic mobility will be retarded as a result. Stacking forces make DNA denaturation highly sensitive to nucleotide sequence: a single nucleotide substitution significantly alters the melting properties and hence the mobility in DGGE. Separation of different homoduplex molecules can be achieved by DGGE, although separation of homo- and heteroduplex DNA is far greater. A major constraint on DGGE is that mutations can only be detected in the lowest melting domain of the frag-

ment because complete denaturation of the molecule retards the mobility sufficiently that no separation of mutant and wild-type molecules occurs. To ensure that the region of interest forms the lowest melting domain, a GC clamp of 20–40 bp is usually added to one end of the fragment to be analyzed (135). The sensitivity of DGGE is in the range of 95–100% (136) for fragments of up to 500 bp.

In classical DGGE, separation is achieved by electrophoresis through a polyacrylamide gel containing a chemical denaturant gradient. Variations on the principle of DGGE include temperature-gradient gel electrophoresis (137) and constant denaturant gel electrophoresis (CDGE) (138). CDGE has been adapted to a fluorescent CE format (139).

The principal disadvantages of DGGE are a relatively low-throughput, complex primer design to include GC clamps in the optimum position and maintain the fragment to be scanned as a single melting domain, and a requirement for extensive optimization for each analysis. Yet its high sensitivity has made it a relatively popular technique within the diagnostic setting.

A temperature-gradient capillary electrophoresis technique that works on the same principle as DGGE has recently been described (140). No prior labeling of the sample is required, and the technique is fully automated for high throughput. 5/5 mutations were tested in a proof of principle, although a full evaluation of the mutation detection efficiency has not yet been made.

#### **6.4. Denaturing High-Performance Liquid Chromatography (DHPLC)**

DHPLC (141), also known as temperature-modulated heteroduplex analysis (TMHA), exploits the differential melting properties of homo- and heteroduplex DNA in order to detect mutations in a manner that has some similarities to DGGE. Differential retention on a chromatography column under conditions of partial thermal denaturation is the physical principle behind DHPLC. Despite its recent introduction, DHPLC has become very popular, and is widely used for both research and diagnostic applications.

Many studies have examined the sensitivity and specificity of DHPLC, and it is clear from these studies that DHPLC is a highly sensitive (91–100% detection) and specific technique (see 122,142–144), although analysis at multiple temperatures may be required for maximum detection (111). The principal advantages of DHPLC are its high sensitivity and high throughput, coupled with minimal post-PCR manipulation and no requirement for sample labeling, although a modification to utilize fluorescent detection has been described (145). Disadvantages include the high capital equipment cost and the need to predict a precise temperature for analysis of each fragment, although theoretical prediction from the DNA sequence is possible (142).

### **6.5. Carbodiimide Modification**

Carbodiimide modifies G- and T-bases that are not base-paired. Its use for mutation detection in mobility shift and primer extension assays has been described (*146,147*) although the method is not widely used.

## **7. Cleavage Methods**

Cleavage methods are able to scan larger fragments than most of the physical techniques, and to identify the location of the mutation in the fragment. For most of the cleavage techniques, a single assay condition is applicable to the analysis of all fragments, whereas many of the physical assays require specific optimization for each different fragment analyzed. Cleavage techniques were originally devised for radioactive labeling, polyacrylamide gel electrophoresis (PAGE), and autoradiography, and can still be used in this format although non-radioactive and/or fluorescent versions of most methods have been described. None of the cleavage methods are now widely used, probably because of the considerable amount of post-PCR manipulation required to generate data.

### **7.1. Chemical Cleavage of Mismatch (CCM)**

Mismatched C- and T-bases can be chemically modified by hydroxylamine and osmium tetroxide, and the modified duplex cleaved at the site of the modification (*148*). The sample to be tested is mixed with a labeled wild-type probe to generate heteroduplexes. For maximum detection, both possible heteroduplexes should be investigated, as modification is restricted to mismatched C- and T-residues. Cleavage products are separated by electrophoresis, with the size of the cleaved product providing the approximate location of the mutation. CCM has an extremely high mutation detection rate of essentially 100% (*149*), although the failure to detect T:G mismatches in some sequence contexts has been reported (*150,151*). CCM is applicable to DNA fragments of 1 kb or longer. However, it has suffered from the disadvantages of being highly laborious and requiring radioactive labeling and highly toxic chemicals for DNA modification, although more recent adaptations to the protocol have addressed many of these problems (*152–155*).

### **7.2. Enzyme Cleavage of Mismatch (EMC)**

The resolvase T4 endonuclease VII introduces double-stranded breaks into duplex DNA at the site of single-base mismatches and small loops (*156*). This activity is used for mutation detection in the enzyme cleavage of mismatch assay (EMC) (*157,158*), also developed commercially as Enzyme Mismatch Detection (EMD). T7 endonuclease I has also been tested in EMC assays (*159*).

Although T4 endonuclease VII shows variable reactivity with different types of mismatches and loop and is also dependent on sequence context, the muta-

tion detection rate of EMC is high—in the range of 91-100% (*160,161*). Like CCM, EMC performs well on fragments of over 1 kb. One drawback of EMC is nonspecific background cleavage, which can complicate interpretation and may obscure genuine results.

More recently, the use of a plant endonuclease, CEL I, in a similar type of assay has been reported (*162,163*). Initial results were promising, and suggested that compared to T4 endonuclease VII, CEL I has more even activity with different mismatches and less nonspecific activity. A high-throughput mutation screening assay utilizing CEL I has recently been described (*164*). It seems that thus far, the ideal mismatch-cleavage enzyme has not been identified, although recently a thermostable endonuclease V has been described that may have potential (*165*). Any enzymatic system must be competitive against increasingly facile physicochemical methods and direct sequencing itself.

### **7.3. Ribonuclease Mismatch Cleavage**

Ribonuclease mismatch cleavage was the first of the mismatch cleavage techniques to be developed. It relies on the ability of RNase A and other RNases to cleave RNA:RNA and RNA:DNA duplexes at or near single-base mismatches (*166,167*). Different mismatches are cleaved with differing efficiency (*168*) with sequence context perhaps accounting for at least part of this variability; small insertions and deletions are also detected (*169*). Detection rates are typically in the range of 60–90% (*170*). Like the other mismatch cleavage techniques, RNase cleavage is able to analyze fragments of up to 1 kb or more (*170*). The major disadvantage of RNase cleavage is the requirement to synthesize RNA *in vitro*. The non-isotopic (NIRCA) format devised by Goldrick et al. has the advantage of requiring no specialised equipment, and is available in commercial kit form and clinical diagnostic applications have been described (*171,172*).

### **7.4. Base Excision Sequence Scanning (BESS)**

Two versions of the BESS technique (also referred to as glycosylase-mediated mutation detection) exist: BESS-T and BESS-G. In the BESS-T reaction, the incorporation of deoxyuridine during PCR, followed by a reaction with uracil N-glycosylase and endonuclease IV, which respectively remove the uracil base and cleave the deoxyribose-phosphate backbone at the abasic site results in the generation of a series of nested DNA fragments, essentially similar to a T-sequencing ladder (*173*). The presence of a mutation is detected as a change to the band pattern in the wild-type, and in this respect is essentially the same as orphan peak analysis. A BESS-G protocol, analogous to BESS-T, uses proprietary reagents to generate a G ladder (*174*). Both reactions must be carried out to be able to detect all possible single-base substitutions.

The original protocol used radioactive labeling, and modification to use fluorescent labeling has been described (174). In most cases, BESS not only identifies the presence and location of a mutation, but also defines the nature of the change to the sequence.

### 7.5. Cleavage Fragment-Length Polymorphism (CFLP)

Cleavase I is a proprietary structure-specific endonuclease that cleaves single-stranded DNA at sites of secondary structure to produce a characteristic pattern of bands for any fragment. Mutations in the DNA fragment result in a change to the band pattern (175,176). Reported mutation detection rates are 92–100% (177) in fragments of up to 550 bp, with indications that fragments of up to 1 kb can be analyzed.

BESS/GMPD and Cleavase do not require the prior generation of heteroduplex DNA, and as a result are independent of sample zygosity. Like BESS/GMPD, Cleavase generates a complex band pattern, and its interpretation is not necessarily straightforward.

### 7.6. MutS

The *E. coli* MutS protein binds to mismatched DNA (178). This property has been exploited in both a gel shift assay (179) and an exonuclease protection assay (19). The latter method reports the position of the mutation, although the sensitivity of the assay has not been established over a large range of samples. Solid-phase immobilized MutS has also been used to detect mutations by binding to nitrocellulose filters (180) or magnetic capture.

## 8. Sequencing Methods

There are two basic sequencing formats in current use: sequencing using dideoxynucleotide chain terminators (181) and the less widely used chemical cleavage method (98). Alternative methods do exist, but sequencing by hybridization (182) has yet to deliver large-scale sequencing; pyrosequencing is making some progress (40) and resequencing by mass spectroscopy requires further improvements of fragment cleavage protocols (24).

Assuming perfect data quality, the Sanger method provides absolute information about the position and nature of a sequence change. It is universally applied in mutation detection for defining mutations identified by scanning techniques, and is generally regarded as the “gold standard” to which other techniques are compared. Sequencing is also widely used as a primary mutation screening technique, which probably reflects the easy commercial availability of the technology together with familiarity with the technique.

The requirements of the human genome project have prompted technological development so that sequencing is now a high-throughput, high-accuracy

technique. The finished human genome sequence has accuracy of 99.99% (183). However, to achieve this, each base has been sequenced on average at least 8–10 times, a depth of coverage not generally used for mutation screening.

Few objective analyses of the mutation detection sensitivity of sequencing have been carried out, partly because of the inherent difficulty in determining the false-negative rate. Several studies have shown that mutation detection rates can be substantially less than 100% (11,58,184,185) and that factors including sequencing chemistry, the nature of the samples analyzed, the depth of coverage and the method of data analysis undoubtedly influence the sensitivity.

For sequencing, as for any method, failure to detect a mutation can occur because the mutation does not generate a difference between wild-type and mutant data, or because the method of data analysis fails to detect a difference that is present. DNA sequencing generates a more significant burden for data analysis than most other scanning methods, because sequencing with both forward and reverse primers, which would be regarded as the minimum acceptable standard for diagnostic work, generates two pieces of data per basepair analyzed, whereas most other techniques generate one or a few pieces of data per fragment analyzed. There are two ways to analyze DNA sequence data: either by visual inspection, which is the only method available for manual gels, and often also used for fluorescent electropherograms. The alternative, which is to use software such as PolyPhred (184) or TraceDiff (186), is only available for automated fluorescent sequencing, and is still dependent on good-quality raw data.

Comparative sequence analysis (CSA) (187), a development of orphan peak analysis (188) is an alternative method of analyzing the products, making a direct comparison of mutant and wild-type sequencing data without the use of base-calling software. Although sensitivity is high and mutations are defined as well as identified, the limitations that apply to sequencing also apply to CSA.

Sequencing of heterozygotes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) has been demonstrated (189). This technique—which is fast, accurate, and fully automated—has tremendous potential for mutation scanning, although current technical limitations on read length must be overcome.

The use of high-density oligonucleotide microarrays for mutation scanning is an application of sequencing by hybridization, which in principle can screen kilobase lengths of DNA for novel mutations with near 100% sensitivity (190). The principle has been tested for the HIV protease, BRCA1, *p53*, and ATM genes, among others (57,191,192). Sensitivity is in the range of 91–99% and is greater for homozygous than for heterozygous changes. Detection of insertion or deletion mutations, especially at repeated sequences remains problematic.



## 9. Protein Methods

A fourth group of methods are those that detect sequence variation at the protein level, either as functional assays or by examining the protein product directly. As a group, these methods are characterised by being highly labor-intensive, with low throughput. However, these disadvantages are offset by the ability to screen large fragments of DNA in a single reaction and obtaining information about the biological consequences of the mutation.

### 9.1. The Protein Truncation Test

The protein truncation test (PTT), also known as the *in vitro* protein synthesis assay (*193,194*) detects mutations which result in premature truncation of translation. Labeled protein synthesized *in vitro* is analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with the presence of a truncating mutation indicated by a change in size of the protein compared to a wild-type control. Sensitivity for truncating mutations is high (reviewed in *195*) with most false-negative results because of mutations at the ends of the fragment. Fragment size for PTT analysis is typically in the range of 1–1.5 kb: for the majority of genes, PTT analysis requires cDNA or large exons as a starting material. The biggest advantage of PTT is that only mutations with a functional consequence, such as truncating mutations, are identified. A yeast *in vivo* assay for truncating mutations, with the ability to screen fragments of up to 3.5 kb has also been described (*196*).

### 9.2. Functional Assays

A small number of assays that directly test protein function from a cloned DNA sequence have been described (*197–199*). Successful applications of functional assays have been described (*see 185, 200*). However, applications for functional assays are limited, not least because of the paucity of information about the molecular function of many disease-associated proteins. A functional assay can only exist when the function of the protein is known; functional protein can be expressed *in vitro* or *in vivo* and a quantifiable assay designed. Many proteins have multiple functional domains: an assay which tests one function does not necessarily test all the functions of the protein; furthermore, functional assays only test nucleotide function at the protein level: nucleotide changes may also have effects on function at the RNA level (*see 201*).

## 10. Summary and Future Developments

To summarize: there are many varied methods available for scanning for unknown mutations, and it is not necessarily a simple matter to select an appropriate method for any individual mutation screening task. The very existence

**Table 2**  
**Methods Currently Used for Primary**  
**Mutation Scanning**

Method	Number of times reported
SSCP	58
Heteroduplex	5
CSGE	8
DGGE	13
DHPLC	16
PTT	8
Sequencing	109
GMPD	1
RNA-SSCP	1
DdF	1
Functional assay	1

of such a wide selection of different methods in itself implies that there is no single ideal method: there may be better or worse choices for the task at hand, but there is rarely a right or wrong answer.

For the period January–June 2001 a survey was made of the method used for initial scanning for novel mutations in papers published in the journals *Nature Genetics*, the *American Journal of Human Genetics*, the *Journal of Medical Genetics*, *Human Molecular Genetics*, and *Human Mutation*. All papers that describe mutation scanning and which specified the technique employed were included, regardless of study size or purpose. When more than one method was used for primary screening, all methods were counted. In total, 185 reports were surveyed.

At present, no mutation scanning method is entirely satisfactory, or meets even current diagnostic demands. Recent trends include adapting existing methods to automated processes using automated data collection and robotic sample handling.

Microarray sequencing, which now exists in a variety of formats, is potentially a tremendously powerful technique. It is capable of far higher throughput than any other, and may be the only technique that can match the demands for sequence variation data generated as a consequence of the completion of the human genome sequence. However, whether the arrays will be read by mass spectroscopy, fluorescence, or some other technique remains to be established. These techniques must compete with microfabricated alternatives to estab-

lished electric field separation technologies (202). Improvements to the sensitivity of mutation detection will inevitably push the burden of genetic diagnostic work into data analysis, and also sample preparation. The probable increase in numbers and types of mutation identified is a potentially valuable resource, not only for the clinical insights concerning genotype and phenotype relationships, but also as part of the ongoing process to document human genome sequence variation. In this regard, it is important that standard nomenclature (203,204) and databases (72,205) are developed to maximize these benefits.

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## Mutation Scanning for the Clinical Laboratory

### *DHPLC*

**John F. Harvey and Julian R. Sampson**

#### **1. Introduction**

This chapter describes the Transgenomic WAVE® DNA Fragment Analysis System with DNA Sep® Technology, as used in our molecular genetics diagnostic laboratories for the detection of unknown mutations. Four software packages are currently available: WAVEmaker 3.4, 4, 4.1, and most recently, Navigator. Because versions 4 and 4.1 are similar, version 4 is presented here. Denaturing high-performance liquid chromatography (DHPLC), also known as TMHA (temperature-modulated heteroduplex analysis) provides a rapid, automated scanning method for mutations, whether known or unknown. Mutations are visualized as a characteristic pattern of peaks corresponding to the mixture of heteroduplexes and homoduplexes formed when wild-type and mutant DNA fragments are hybridized (**I**; **Fig. 1**).

Individuals who are heterozygous for a mutation or polymorphism have a 1 : 1 ratio of wild-type and mutant DNA. A mixture of hetero- and homoduplexes is formed when the polymerase chain reaction (PCR) product containing the mutation is heated to 95°C and slowly cooled. To form these molecular species, the DNA from individuals who have two mutant alleles (homozygous mutation) must be mixed with the corresponding wild-type DNA fragment and hybridized. After this treatment, a sample will contain a mixture of hetero- and homoduplexes. However, DNA from homozygous wild-type individuals will form only one species, the homoduplex wild-type, unless mixed as described previously.

The WAVE nucleic acid analysis platform is based upon ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC), and can be

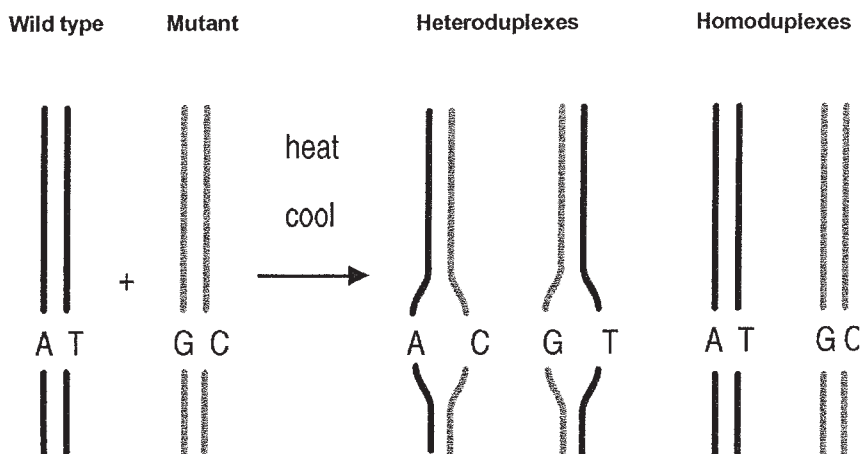


Fig. 1. Formation of a 1:1 mixture of heteroduplexes and homoduplexes through hybridization.

operated in three modes, as determined by the temperature at which separation is performed (**Fig. 2**).

The process has two phases—a stationary phase consisting of a DNA Sep<sup>®</sup> column packed with alkylated nonporous polystyrene-divinyl benzyl C18 and a mobile phase of chemicals including acetonitrile (ACN), (an organic co-solvent that is used to selectively elute samples) and the ion-pairing agent triethylammonium acetate (TEAA), which ensures that the DNA interacts with the stationary phase. Under non-denaturing conditions (<50°C) double-stranded DNA (dsDNA) will be eluted according to size with the WAVE buffer system (**Fig. 2A**). Essentially, the DNA interacts electrostatically with the ammonium ion of the TEAA on the basis of size. The larger the DNA, the more interactions it has with TEAA. Once it is associated with TEAA, it effectively has a hydrophobic outer coating that allows it to interact with the hydrophobic DNASep column, and the number of bonds formed are determined by the DNA fragment size. The TEAA acts as a bridging molecule, and the alkyl chains of the TEAA interact with the hydrophobic surface of the DNASep matrix. Elution of DNA from the DNASep matrix occurs through the use of the hydrophobic eluant ACN, which reduces the hydrophobic interaction between the stationary phase and the alkyl chains of the bridging molecules. As a gradient (a constantly increasing concentration) of ACN is run through the column, DNA fragments of increasing size are eluted. Under non-denaturing conditions (50°C), this separation is size-dependent but not sequence-dependent (**Fig. 2A**; *see Note 1*).

At fully denaturing temperatures, single-stranded molecules (RNA or DNA) can be separated (**Fig. 2C**). This mode can be used for oligonucleotide quality

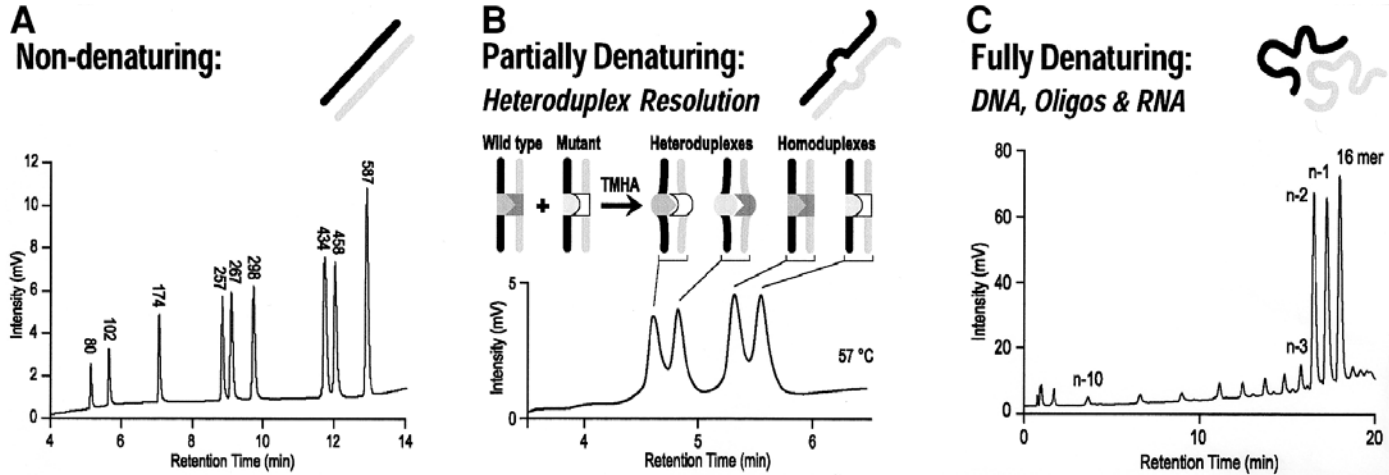


Fig. 2. Three modes of operation of the WAVE Nucleic Acid System. WAVE traces of: (A) a pUC18 *Hae*III digest, (B) a sample heterozygous for an A to G transition in a 209-bp *DYS271* nucleotide sequence, (C) the separation profile of 14-, 15-, and 16-mer oligonucleotides.

control and purification. Oligos can be effectively separated from failure peaks using a fragment collector (*see Note 2*).

The separation of fragments under partially denaturing conditions is the focus of this chapter. Denaturing and hybridizing a sample containing two products that differ by as little as one nucleotide results in the formation of two homoduplexes and two heteroduplexes. These may be visualized as four distinct peaks (**Fig. 2B**), but any difference in peak profile compared with a homozygote wild-type control is indicative of a mutation (*see Note 3*).

At a column temperature determined by use of the melting algorithm WAVEmaker software (Transgenomic Ltd., Crewe, UK), DHPLC separates the DNA molecules, with the heteroduplexes eluting first (**Fig. 2B**). This is the result of bubble formation in the dsDNA duplex, which reduces the number of charges accessible for electrostatic interactions between the DNA bases and the ion-pairing layer of the column stationary phase. The charge density of the DNA molecule is reduced within the single-stranded region.

The major procedures involved in DHPLC mutation detection include: PCR primer design (*see Note 4*); oil-free PCR (*see Note 5*); heteroduplex formation; melting profile determination of the fragment; method preparation, which includes setting of the separation gradient; making a new project, which includes sample sheet preparation, sample running, and sample analysis. When optimized DHPLC provides a reported sensitivity of 94–100% for the detection of heterozygous sequence variants, comparing well with full-sequence analysis (2–9) (*see Note 6*). DHPLC has been shown to detect mosaic mutations that account for only a few percent of alleles in a PCR product and that remain undetected by direct sequence analysis (10). The system is gel-free and can be mostly automated, since samples are transferred directly from the PCR cyclor to the WAVE platform.

## 2. Materials

### 2.1. PCR Amplification

1. DNA template ~50 ng per reaction (*see Note 7*).
2. Optimase™ Polymerase (Transgenomic Ltd., Crewe, UK) Amplitaq Gold™ (Perkin-Elmer, Foster City, CA) (*see Note 8*).
3. GeneAmp® 10X PCR buffer 11 (Perkin-Elmer) (*see Note 9*) which contains 100 mM Tris-HCl pH 8.3, 500 mM KCl. Store at –20°C for long-term storage and 4°C for daily use or short-term storage.
4. 25 mM MgCl<sub>2</sub> solution (Perkin-Elmer). Store at –20°C long term and 4°C short term (*see Note 10*).
5. 100 mM stocks of deoxyadenosine triphosphate (dATP), thymidinetriphosphate (TTP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP) stored at –20°C. Dilute to yield a 2-mM mix for daily use and store at 4°C (Promega, Madison, WI).

6. Primer stocks at 100  $\mu\text{M}$  stored at  $-20^{\circ}\text{C}$ . Working stocks of 10  $\mu\text{M}$  stored at  $4^{\circ}\text{C}$ . (ThermoHybaid, Interactiva Division, Germany. <http://www.thermoHybaid.com>).
7. Thermal cycler allowing oil-free operation and 96-well plate format, e.g., 9600 (Perkin-Elmer) or PTC 225 Tetrad (GRI, Essex, UK).
8. Microtiter plates or tube strips. Our plates consist of either 8 or 12 0.2-mL PCR tubes (Strip-ease, GRI) held in a MicroAmp<sup>®</sup> 96-well tray/retainer system (Perkin-Elmer) or 96-well halfskirt plates (Axygen, Thistle Scientific, UK) sealed with adhesive PCR film (ABgene, Surrey, UK).

## 2.2. DHPLC and Data Analysis

1. WAVE<sup>®</sup> DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE) (*see Note 11*).
2. DNASep<sup>®</sup> cartridges (Transgenomic Ltd.) (*see Note 12*).
3. In-line filters (Transgenomic Ltd.) (*see Note 13*).

## 2.3. Water and Buffers (*see Note 14*)

The buffer solutions for the WAVE should be made to analytical chemistry standards, using quality-assured glassware. For health and safety reasons, always wear nitrile gloves when making buffers, and use a fumehood when available. **Caution:** for safety and disposal, *see Note 16*. Whenever possible, use water of the highest quality, e.g., Millipore (Millipore Co, Bedford, MA), since transition ions shorten the column's life. The minimum standard is HPLC-grade water (Sigma Aldrich Co Ltd., Poole, UK). Before use, the Duran 1-L or 2-L bottles (Sigma Aldrich Co Ltd.) we use for buffers are washed out with Millipore water (to remove any transition ions), especially if the glassware has been autoclaved.

1. Buffer A: 0.1 M triethyl ammonium acetate (TEAA) for ion pairing (Transgenomic Ltd.). Store 2 M stock at  $4^{\circ}\text{C}$ .
2. Buffer B: 0.1 M TEAA and 25% acetonitrile (ACN) (Sigma Aldrich Co Ltd.) for producing the gradient.
3. Buffer C: 75% ACN (Sigma Aldrich Co Ltd.) for cleaning the column.
4. Buffer D: 8% ACN for washing the needle.

## 2.4. Quality Control (*see Note 15*)

1. A pUC18 HaeIII digest (Transgenomic Ltd). Store at  $-20^{\circ}\text{C}$ .
2. The mutation detection control, DYS271. This is a 209-bp heterozygous locus of a G > A transversion (Transgenomic Ltd.). Store at  $-20^{\circ}\text{C}$ .

## 3. Methods

### 3.1. PCR Amplification (*Table 1*)

Carry out the PCR reactions in a total vol of 25  $\mu\text{L}$ , or 50  $\mu\text{L}$  if more than two injections.



**Table 1**  
**Reagent Volumes for a Single PCR Reaction**

	X1 reaction
Gene Amp 10X buffer 11 or Optimase reaction buffer 10X	2.5
2 mM deoxynucleotide 5' triphosphate (dNTP) mix	2.5
25 mM MgCl <sub>2</sub>	2.5
10 μM Primer—Forward	1.0
10 μM Primer—Reverse	1.0
AmpliTaq Gold™ or Optimase	0.1
Sterile distilled water	10.4
50 ng DNA	5.0

Prepare a master mix based on the volumes listed here, allowing for 10% possible pipetting wastage, and remembering to allow for the equivalent number of wild-type samples if mixing is necessary, as when screening males for an X-linked condition.

Amplify the samples under appropriate conditions, including an initial denaturation step at 95°C for 15 min, and then 35 cycles of denaturation, annealing, and extension followed by a final extension at 72°C for 10 min (*see Note 5*).

For a full plate, aliquot the master mix into each vial of a 12-vial strip-ease (GRI, Essex, UK) (*see Subheading 2.1.8.*).

Add primers as appropriate, and distribute the mix using a multichannel pipetter. Patient DNA can then be added in a similar way, allowing 50 ng DNA per well. Include normal, mutation-positive, and “no DNA” controls in the setup design.

### **3.2. Heteroduplex Formation**

Directly before analysis, heat the PCR products, and then cool slowly to promote heteroduplex formation: heat for 5 min at 95°C, then reduce temperature at 1.5°C per cycle for 45 cycles. To detect homozygous mutant DNA and X-linked mutations in males, mix the patient's sample with the corresponding wild-type PCR product in a ratio of approx 1 : 1 prior to the heteroduplex step. It is advisable to first check all PCR products on a gel to ensure that there are no failures.

The total volume for reaction may need to be increased if there are more than three injections per sample, as approx 5–8 μL are required per injection. Plates may be subject to evaporation loss during the run. This can be minimized by adjusting the cooling of the chiller block of the autosampler. Alternatively,

“easy pierce” adhesive foil to cover the plates (ABgene, Surrey, UK) (*see Note 17*). Subsequently, the plates can be resealed with adhesive plastic film (ABgene) if a sample is required for further analysis.

### **3.3. Separation Gradient Determination**

For gradient optimization, you will need about 50  $\mu\text{L}$  of the wild-type PCR product of length 60–500 basepairs (bp), prepared under the same conditions as those used for diagnostic samples. Mutant samples are not needed for this optimization.

### **3.4. Size and Purity Determination**

Determine the fragment size and purity of the PCR product under non-denaturing conditions. It must provide a single sharp elution peak of the expected size. If more than one peak is present, or a peak with a shoulder, then there is more than one fragment of DNA in the sample, and the PCR protocol may need to be optimized.

### **3.5. Selection of Gradient for Mutation Detection**

The recommended gradient for mutation detection is a slope of 2% increase in buffer B per min. The universal gradient illustrated in **Table 2** can be used for unknown sequences or any sample within the size range that is amenable to the technique, although it has not been optimized for speed of analysis. Run times can be reduced by starting the clean off at a lower percentage of buffer B. This optimization is performed by the WAVE<sup>®</sup> system utility software. Run the gradient initially at non-denaturing conditions of 50°C.

### **3.6. Separation Temperature**

Typically, at the  $T_m$  (the temperature at which fragments have an average helicity of 75%), mutations are detected at a retention time of 0.75–1 min earlier than the elution of the fragment under non-denaturing conditions (50°C) using the universal gradient (equivalent to 1.5–2% less buffer B).

### **3.7. DHPLC and Data Analysis**

There are three main sections to the WAVEmaker software:

1. D-7000 Admin: This is the filing system containing application folders in which data is stored. Make a new application folder every month and copy methods across from other folders. The programs will not run if more than one folder is open. The authors store application folders in the “D” drive.
2. D-7000 HSM: This is the main program, which is icon-driven and can be followed easily in a stepwise fashion.

**Table 2**  
**Universal Gradient for Mutation Detection**  
**on the WAVE® System**

Time (min)	%A	%B	Flow (mL/min)
0.0	65	35	0.9
0.1	60	40	0.9
16.1	28	72	0.9
16.2	0	100	0.9
16.7	0	100	0.9
16.8	85	15	0.9
18.0	85	15	0.9

3. WAVEmaker: This is used for designing methods before importing the new method into D-7000 HSM (this is done by the operator in version 3.4, but is automatically imported by the software in version 4).

For new users of WAVEmaker 4.0 in our laboratory, we have designed a stepwise program of operation, although a more detailed training protocol in procedures and machine operation will be given by the manufacturers on machine purchase.

### 3.7.1. Making a New Project in WAVEmaker 4.0

1. Import a new sequence: Import your sequence as a text file, which WAVEmaker changes to a .DNA file. Save the DNA file in your sequence folder. It is then ready to be used in a project. Import your sequence from disk by going to:

***File → Open DNA sequence → Select your sequence from your disk***

WAVEmaker will say that the file will be renamed “***your sequence .DNA.***” Click on OK. Your sequence now appears in the drop down list under the “***Sequence file***” box in the project. Now save your sequence as a .DNA file in your sequence folder. Go to:

***File → Save DNA → Name your sequence and change → Save in your sequence as the “Save as type” box to Sequence file (\*.DNA) sequence folder***

Your sequence is now saved as a .DNA file in your sequence folder.

2. Using a previously saved sequence: Sequences previously saved in your sequence folder as a .DNA file are ready to use in a project. To open the sequence in the new project, go to:

***File → Open DNA sequence → Select your sequence from your sequence folder***

Your sequence will now appear in the drop down list under the “*Sequence file*” box in the project (see **Note 18**).

### 3.7.2. Melting Profile

1. Making a melting profile (**Fig. 3–5**): Once you have your sequence in the drop down list under the “*Sequence file*” box, click on the sequence name to make it appear in the “*Sequence file*” box. To check that the sequence is correct and to calculate the  $T_m$  of your fragment (denoting 75% helicity), click on the “*Sequence*” button on the left of the screen. You will now see your sequence displayed in the text box. The  $T_m$  for your fragment can be seen to the right of the text box.

To see a melting profile of your fragment, click on the “*Melting*” button on the left of the screen and then click on “*Calculate*” on the right of the top box. This will show you “*helical fraction vs temp.*” To see a melting profile of your fragment at a given temperature, click on “*Calculate*” to the right of the lower box. The temperature range of the profile can be changed in the boxes above the “*Calculate*” button (see **Note 19**).

2. To print out a melting profile: Print out the melting profile for your fragment by going to:

*File* → *Print report*

You will then see a preview of your report. Click on the “*print*” button to print out your report.

### 3.7.3. Method

1. To make a new method: Open your sequence from the drop down list under the “*Sequence file*” box. The name of your sequence will now appear in the box. Enter the application type (usually “mutation”) either by clicking on the appropriate circle or clicking on “*Mutation*” on the drop down list under the “*Application type*” box. The default values calculated for your fragment are then shown in the boxes.

Click on the “*Gradient*” button on the left of the screen to display the gradient information. Change the default settings displayed for use with (or without) the accelerator, which will decrease the run time for each sample from 6.8 min to 5.6 min (see **Table 3**). To do this, go to the menu at the top of the screen and click on:

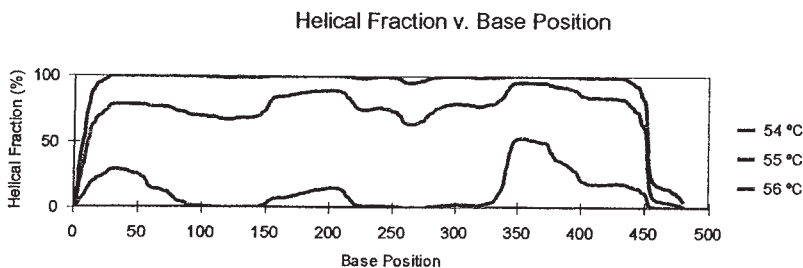
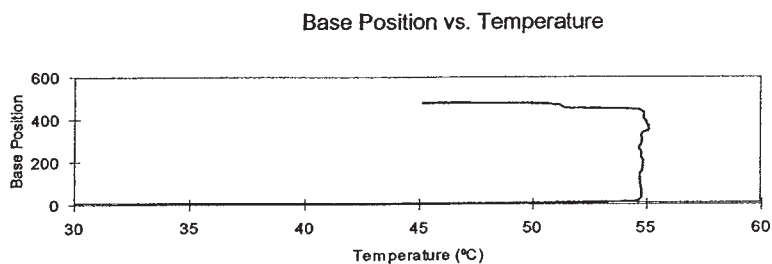
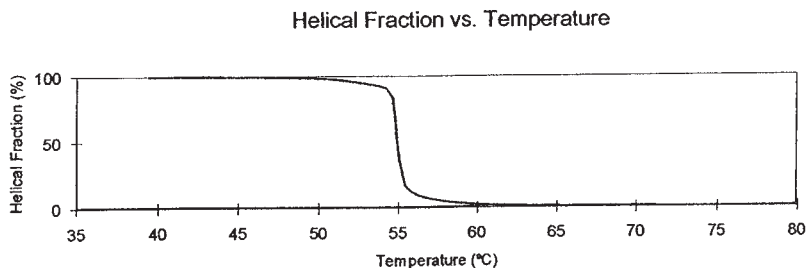
*Project* → *Project options* → *Then click on the “Gradient” tab*

Check the “*Update changes to all samples*” and “*Save project options as default*” boxes. Click on “*OK*.” This should change the run time to 5.6 min on the gradient display (with accelerator) and 6.8 min (without accelerator).

Default values are shown in the oven temp (–) and time shift (0) boxes. If the “*Oven temp*” and “*Time shift*” boxes need changing, click on the relevant box and enter the value you want followed by “*Enter*.” You should see the red and blue lines on the gradient profile shift when you press “enter.” It is important that

### Melting Curves Report

Project: C:\Program Files\Transgenomic\WAVEMaker 4.0\Sequences\Rachel\Wave Round 2\Untitled.wmp  
 Sequence Name: Ac6054-1  
 Sequence Desc:



```
CTGATGCAATCAGACAAGACAAGAAAGGTCCTCCTGAACTGGAAAAGGAGAAGTCAAATTTATTCCTCTTTCGATGATGATGATCTCATATTTGGAAATACCTTAAGA
CTCTATCAAAAATTTGGAACTGATAAAGCAAATTAAGTAAAGTTGCAGGATACAAAATCAACATACGAAAATCAGTAGCATTCTGTATGCCAACAGCAGACAATCGA
AAAAAATTTAAAGTAAGCCATTITGCAATAGCAACAATAAAATTAATACTTATGAGTTAACCAAAGAACGAAATGACCTCTACAAGGAAAACATAAAAACTGATA
AAAGAAATTTAAGAGGACACACACGAAATGGAAAAGATAGGCCATGTTTATGGATTGGAAGAATCGATATGTTAAAAATGTTGATCACTATCCAAAGCAATCTGCAGAT
TCAATGCAATCCCTATTAATAATCAAAATGACATCTTCACA
```

Fig. 3. Melting curves report for a 480-bp DNA fragment showing a single melting domain with a predicted  $T_m$  of 55°C.

you see the change on the gradient profile in order for it to be saved correctly in your method.

If all the data for your method is correct, save it in your method folder. Go to:

**File** → **Save method as** → **Name your method** → **Save in your method folder**

**Melting Curves Report**

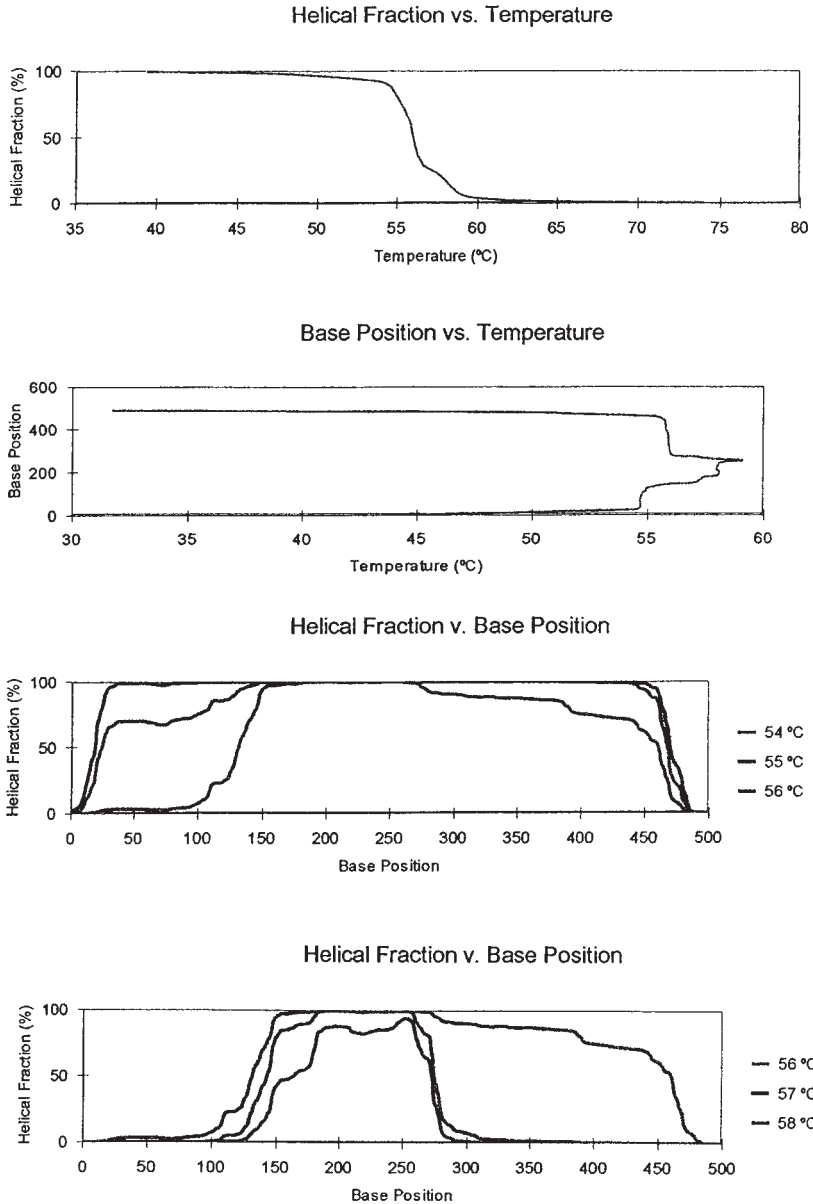


Fig. 4. Melting curves report for a 492-bp DNA fragment showing two melting domains with predicted  $T_m$  of 55°C and 58°C to accommodate a GC-rich domain. A T/G transversion at base position 123 was detected in this fragment at the predicted  $T_m$  of 55°C (Fig. 5).

**Table 3**  
**Gradient Settings for Mutation Detection**

	With Accelerator	Without Accelerator
Caution level	Normal	Normal
Drop for loading	5% B	5% B
Loading duration	0.1 min	0.1 min
Gradient duration	4.0 min	4.0 min
Clean duration	0.1 min	0.5 min
Equilibration duration	0.9 min	2.0 min

Methods are usually named “Your fragment@temp” e.g., “Exon 6@57.” Your method name is displayed in the “*Method name*” box and your method saved in your method folder.

2. To open a previously saved method: Open a method already saved in your method folder by going to:

*File* → *Open method* → *Select your method from your method folder*

Your method appears in the drop down list below the “*Method name*” box.

### 3.7.4. Project

1. To make a new project: You can open your previously saved sequence and method (if not already open) by going to:

*File* → *Open DNA sequence/method* → *Select your sequence/method from the appropriate folder*

The method data for your fragment is displayed in the boxes. Click on the “*Sample table*” tab. Your method data is displayed in the top line of the sample table. To enter the same method for additional samples, highlight the top line and drag it down to highlight the number of samples you need. Move the cursor into the green highlighted area and right click on the mouse. When the menu is displayed, click on “*Fill*” and wait.

Eventually, your method data will be shown in all of the lines you have selected. The vial numbers of the samples may need changing if you are not starting at the beginning of a plate. Enter your sample names, typing the name in the “*Sample name*” box or copy and paste from a previous project.

Check that all the data for each sample is correct for your method. Repeat the process for any additional methods to be run in the same project. If not using an accelerator, a 45-min column wash at 0.9 mL/min with buffer C (75% ACN) is included at the end of each run series followed by the low flow settings (A and B 50:50, at 0.5 mL/min).



## Chromatogram Report

Project: E:\Rachel\February\AL9048-1.wmp  
 Application Folder:  
 Data Path: C:\Wn32App\HSM\February

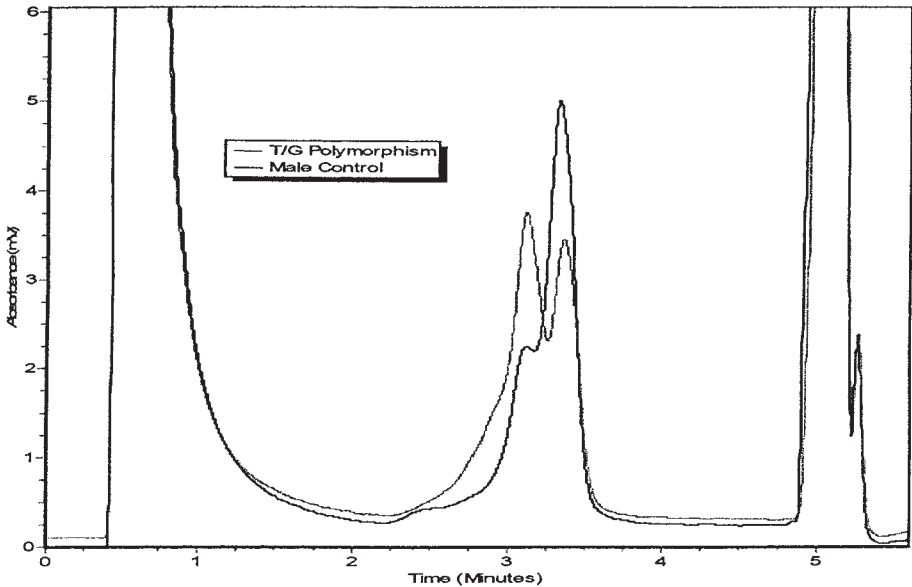


Fig. 5. WAVE trace of a T/G transversion (double peak) found at  $T_m$  55°C in the 492-bp fragment of Fig. 4. A normal male control (single homoduplex peak) is superimposed for comparison. Peaks at the beginning and end of the trace represent the injection peak and accelerator peak, respectively. The mutation was characterized by fluorescent sequencing using an ABI 377. Data courtesy of Rachel Wycherley, National Genetics Reference Laboratory (Wessex) UK.

Once your sample table is complete, check that the default “*Application folder*” and “*Column name*” displayed at the bottom of the screen are correct. Save your project by going to:

**File** → **Save project as** → **Name your project** → **Save in your project folder**

Projects are usually named “Gene fragment@temp date”—e.g., “APCExon6@57 28.02.01.” It is important that you put the date at the end of your project name if projects are archived by month.

2. To open a previously saved project: If your project has been saved in your project folder, go to:

*File* → *Open project* → *Select your project from your project folder*

When you click on “*Open project*” WAVEmaker will ask if you want to “*Save the current project?*” If you have just opened WAVEmaker and this is a blank project, then click on “*No*” or you will save the blank project in place of your completed one. If this happens, your original project cannot be retrieved.

When you next select a project from your project folder, the method and sample table will normally be displayed as previously saved, but these should be checked before starting a run.

### 3.7.5. To Run Samples

1. Starting a run: Before starting, check that all buffer and wash solution bottles are at least half full. Always wash the injection valve and needle and purge liquid lines according to the manufacturer’s instructions. Once your project has been opened/saved, go to the bottom of the sample table and click on “*Run samples*” to start the new run. The estimated run time will be displayed at the bottom right portion of the screen. Once the run has started, the display will change from the sample table to the monitor view, where you will see the result as your samples are being run.
2. To alter a run once started: It is possible to change your method data while the run is in progress. To do this, go to the sample table and click on the sample you want to change. Fill in the new data, and your run will be altered accordingly. Once a sample is in the process of running, its record will turn gray on the sample table, and changes cannot be made.
3. To stop a run once it is started: If you need to stop or pause your run once it has started, go to the sample table view and click on “*Pause table.*” Doing this means, you will lose your result for the sample in progress if it is not complete. Your run can now either be restarted by clicking on “*Resume table*” or stopped by clicking on “*Abort.*”

### 3.7.6. Results

1. To view results while WAVEmaker is running: You can look at and print out your results while a run is in progress. To do this change from the monitor view to the results view by clicking on the “*Results*” tab at the top of the screen. You have a choice of display settings for your results, and you can either see them in a single window or as two smaller windows. To change the display from two windows (default) to one, click on the first of three buttons showing a peak and a number. This button has a number one on it, and is just above the list of samples. To show your results, just double click on the sample you want to see from the list on the left of the window. The result is then displayed in the window.
2. To change the display settings: If you want to change the display settings for the window (e.g., to alter the axes or to add a title), move the cursor onto the window and right click the mouse. On the menu that appears, click on “*Chart.*” You can now change any of the settings. Once done, click on “*Apply*” to get a preview of

- the changes and then **“OK.”** If you change the axes settings, then you must check the **“Save axes range as default”** box to apply the changes to all of your results.
3. To display more than one result: It is possible to see more than one result displayed in the same window. To do this, the **“Show all”** box just above the upper results window must be checked. Once this is done, go to the list of samples and double click on the ones that you want to display. The gap between each result displayed can be changed by moving the cursor onto the result window, right clicking on the mouse, and then choosing **“Chart”** from the menu. You will need to change the **“Trace axes offset”** setting to make the gap between individual results larger or smaller.
  4. To review the results one at a time: Your results can be seen as a review using the up and down arrows rather than double clicking on each sample name. To do this check the **“Review box”** above the upper result window. To review your results, double click on the first sample you want to display in the result window, and then use the up and down arrows to scroll through your results.
  5. To print out results: When your results are ready to be printed out, go to:

**File → print report**

This will show you a preview of your report before you print it out. To print the report, click on the **“Print”** button. Your sample table and gradient information can also be printed out in the same way as your results. To do this, check the appropriate box on the left side of the screen and print as before.

### 3.7.7. Archiving

At the end of each month, it is a good idea to archive your projects for that particular month into a monthly folder. This is made easier when you have named your projects using the date that they were run.

### 3.7.8. A WAVE System Report

Printing out a WAVE system report may be useful if there is a problem with the WAVE as it plots pressure vs temperature for the pump. Go into D-7000 HSM. Click on the **“blue filing cabinet”** icon on the left of the screen and select the appropriate application folder. Click on the **“chromatogram”** icon (also on the left of the screen). This gives you a list of samples you have run in that application folder. Click on the sample you want to print out a report for. You should see another window with the name of that sample in. Click on the **“recalculate”** button at the bottom of the screen followed by the **“modify report”** button. This should show you a print preview of your WAVE system report.

## 4. Notes

1. This mode works well for quantitative work comparing peak height or area ratios for duplication/deletion detection (**11**).

2. There are a range of columns and buffers available (Transgenomic Ltd.) for high purification work (Transgenomic application Note 103).
3. In practice, a four-peak pattern is seldom seen, as the homo- and heteroduplex elution profiles associated with many mutations overlap. A distinct mutant pattern is nevertheless usually seen when the fragment-specific  $T_m$  (temperature at which ~75% helical) is selected for analysis and at temperatures corresponding to ~70–90% melt.
4. Many operators will choose to use primer sets that were originally designed for gel SSCP/heteroduplex analysis. However, consideration should be given to the predicted fragment melting profiles.

For the detection of unknown mutations, it is probably best to select fragments of 150–450 bp (although single-base mutations have been detected in 1.5-kb fragments using the technique described here). If the sequence is known, construct the melting profile using appropriate software. Locate primers near the high points on this profile so that GC-rich regions are at the ends of fragments rather than in the middle. Avoid the inclusion of regions that differ by more than 15°C in the same fragment. If this is unavoidable, it may be necessary to substitute dGTP with N<sup>7</sup>-dGTP (7-deaza-2'-deoxyguanosine 5'-triphosphate) to lower the melting temperature of GC-rich regions, or exceptionally, to include a short GC clamp of three or four bases. Once the approximate locations of primers have been determined, more detailed primer design can begin.

The use of primers with non-template tails such as universal sequencing primers or T7 promoters should be avoided, although it may be possible to use these with *Pfu* polymerase (Stratagene, La Jolla, CA) or Optimase (Transgenomic Ltd., Crewe, UK). The recommended primer melting temperature  $T_m$  is 56°C. The difference in  $T_m$  between Primer 1 and 2 should be less than 1°C. The difference in  $T_m$  between primer and template should be about 25°C. Any possible primer dimers should be less stable than the 3'-pentamer by at least 5 kcal/mol. Any primer self-annealed loops should have a  $T_m$  of less than 12°C. Primers should be of high purity without failure sequences. To avoid degradation, it is preferable to store primers in Tris-HCl (pH 8.0) buffer rather than in pure water. Many web sites are now available for effective primer design—e.g., Primer 3 software program ([http://www.genome.wi.mit.edu/genome\\_software/other/primer3/htm](http://www.genome.wi.mit.edu/genome_software/other/primer3/htm) and [www.mutationdiscovery.com](http://www.mutationdiscovery.com)).

5. The PCR must be oil-free. Although the authors use standard PCR procedures, “touch down” PCR may be used to minimize misprimed products. This is particularly recommended by the manufacturers if using *Pfu* polymerase (Tables 4 and 5).
6. Although elution profiles are consistent for a specific mutation, different mutations may share indistinguishable profiles. Sequencing is advised for characterization of variants. DHPLC and sequencing analyses provide an excellent two-tiered approach to mutation analysis. However, specific mutation analysis can be achieved by DHPLC using primer extension methods (12).
7. High template concentrations can affect the WAVE trace.

**Table 4**  
**Protocol for PCR Amplification with Pfu**

Protocol for Pfu	Initial	Touchdown	Main amplification	Final
Denaturation	95°C, 45 s	94°C, 45 s	94°C, 45 s	
Annealing		63°C, -0.5°C per cycle for 14 cycles to 56.5°C, 45 s	56°C, 45 s	
Extension		72°C, 2 min	72°C, 2 min	72°C, 10 min, finally perform heteroduplex step
Cycles	1	14	20	1

**Table 5**  
**Protocol for PCR Amplification with Optimase or TaqGold™**

Protocol for Taq or TaqGold™	Initial	Touchdown	Main amplification	Final
Denaturation	95°C, 5 min (15 min for TaqGold™)	94°C, 30 s	94°C, 30 s	
Annealing		63°C, -0.5°C per cycle for 14 cycles to 56.5°C, 1 min	56°C, 1 min	
Extension		72°C, 1 min	72°C, 1 min	72°C, 5 min, finally perform heteroduplex step
Cycles	1	14	20	1

8. The recent introduction of Optimase (Transgenomic, Ltd.) with its own proof-reader is giving favorable results in our labs. Amplitaq Gold (Perkin-Elmer) works well for smaller fragments up to 300 bp, yielding fewer artefacts than standard Taq polymerase or Hot Start Taq. Although we do not use Pfu polymerase to generate PCR products for DHPLC in our laboratories, this enzyme minimizes PCR-induced mutations (Taq can cause 15% of dsDNA fragments to contain one or more mutation). As a rule of thumb, manufacturers recommend the use of Taq only if cycle number  $\times$  base pairs  $< 8000$ . Pfu is expensive, and some laboratories use a

Pfu: Taq Gold mix to minimize cost. Optimase appears to be a cheaper, effective alternative. The use of a proofreading enzyme is recommended for fluorescence work on the WAVE, and the increased sensitivity of fluorescence (100×) can introduce artefacts if using Taq polymerase.

9. If other buffer systems are used containing additional reagents such as detergents, it is important to check first with DHPLC column manufacturers (Transgenomic) to ensure that they are compatible with the column packing, otherwise column life can be seriously curtailed. In our experience, dimethyl sulfoxide (DMSO) and 7 deaza-2-dGTP do not cause problems.
10. PCR amplification items 2, 3, and 4 can be purchased together as a kit from Perkin-Elmer.
11. The integrated system is comprised of an autosampler, pump, column oven, DNASep<sup>®</sup> column, in-line degasser, UV detector, WAVE utility software 3.4, 4.0, 4.1, or Navigator computer system, monitor, and color printer. Optional extras include a fragment collector for isolating individual peaks for further analysis or sequencing (Transgenomic Application note 104), an accelerator for reducing the time of sample throughput—e.g., from 6.8–5.6 min. The accelerator may be a useful addition for high-throughput work but it needs careful optimizing and current methods must be modified to ensure that peaks are captured in the smaller window that is provided. An additional facility is a fluorescence detector for increased sensitivity (×100). Currently, only a single-channel detector is available, but development of multichannel systems will allow sample multiplexing and thus higher throughput, although at extra cost for fluorescently tagged primers. The latest high-throughput WAVE<sup>®</sup> system (3500 HT) has a 2 × 96-well capacity sample plates with 2 × 384-well sample capacity, and incorporate a wide bore column, allowing an increase of standard flow rate from 0.9 mL/min to 1.5 mL per min, reducing the run time per sample to approx 3.2 min. The new WAVE-MED is a cheaper and currently slower machine using a single gradient and disposable column.
12. Enzymes, multivalent ions (such as Mg<sup>2+</sup>) and larger DNA fragments are present in all PCR samples. They are injected together with the PCR product on the WAVE<sup>®</sup> DNA Fragment Analysis System. They are usually eluted from the DNASep cartridge during the washing step with 100% buffer B (0.1 M TEAA, 25% ACN). However, some of those substances can accumulate on the surface of the separation matrix over time, causing back pressure and a loss of separation performance. The DNASep cartridge can withstand pressures of up to 3600 psi, and usually lasts for 4000–6000 injections or considerably more if treated carefully.
13. Replace the in-line filters every 1500 injections, and keep a log. It is also important to keep a log of injection numbers and the column life to highlight problems. Any maintenance carried out by the user should also be recorded.
14. The most common source of error is in the preparation of the buffers. Prepare these carefully, using volumetric flasks rinsed in HPLC water. The authors use water from a Millipore Elix 3 reverse osmosis system (Millipore Co., Bedford, MA). Water is taken from this and then polished by taking through a Simplicity 185

(Millipore) column for immediate buffer preparation. The quality of the water deteriorates if it is not used immediately. Ready-made buffers may now be purchased from Transgenomics, Ltd. This is recommended and eliminates in-house preparation variables.

Buffers A, B, and D must be changed weekly, and buffer C every 3 wk if not used. We find it convenient to keep a WAVE logsheet for each month, including date; buffer change; A B C D; QC run; injection number; notes.

15. In accordance with Best Practice Guidelines (**13**) mutation standards should be run weekly and after any system change for good internal quality control. If a QC test fails (that is the resolution and sensitivity has dropped appreciably), try an 80°C column hot wash. If this fails, an 80°C reverse hot wash with buffer C.

For the 80°C column wash, set the oven to 80°C and run 100% buffer C through for 30 min at 0.9 mL/min. Set the oven back to 50°C, allow to cool, and then run buffers A and B 50:50 at 0.9 mL/min for at least 3 h to equilibrate. Return to low flow and run two blank samples, then a pUC18 digest plus the mutation standard. If there is no improvement, try the reverse hot wash. First switch off the pump and reverse the orientation of the column, then proceed as previously by setting the oven at 80°C and running at 100% buffer C for 30 min at 0.9 mL/min flow and set the oven back to 50°C. Once the oven has cooled to about 60°C, return the column to its original orientation and equilibrate as before. Run through two blanks followed by the rest (pUC18 digest and mutation standard). If there is still no improvement, remove the in-line filter, replace with a plastic union joint, and run standards. If the problem is resolved, replace with a new in-line filter. Oven calibration may be suspect if DYS271 heterozygous/homozygous peak distances are not equivalent. If the problem remains, change the column and/or call/E-mail the Transgenomic Help Desk:

Tel: 1 (888) 233-WAVE  
Fax: 1 (402) 733-1932  
E-mail: [custserv@transgenomic.com](mailto:custserv@transgenomic.com)  
or UK E-mail: [help.desk@transgenomic.co.uk](mailto:help.desk@transgenomic.co.uk)

16. TEAA and particularly ACN are hazardous chemicals, and safety procedures as outlined in the manufacturer's instructions should be observed. Ideally, buffers should be prepared in a fume cabinet. A relatively large volume of waste buffer accumulates. To collect the waste, we use 25-L containers (placed under the machine bench) provided by a local disposal firm that provides containers (and labels), and collect them every 2–3 mo. The waste contents and quantities (to the L) must be recorded for disposal. Many hospital departments already use disposal contractors, and it is usually possible to link to an existing service. Many WAVE® users rely on a well ventilated laboratory to remove ACN fumes. We also cover the opening of the waste container with parafilm "M" (Sigma Aldrich Co Ltd.). Ideally, the waste could be vented to a fumehood. As an alternative, we are currently attempting to improve our present collection system by introducing a carbon filter to vent the waste containers.



17. A number of different types of plate covers are available. Blockages with foil plugs or build up of adhesive on the needle can cause a problem. It is advisable to wipe the needle at least every 1000 injections with IPA or equivalent to remove adhesive. Transgenomic (Crewe, UK) have trialed a number of covers, and can advise as appropriate. Sharp needles are available from the manufacturer, and may alleviate the problem.
18. Once you are familiar with WAVEmaker 4, there are a number of shortcuts you can use when you are making your project by clicking on the icons at the top of the screen. For example, instead of going to “File → Open DNA sequence” and then looking for your sequence, you can just click on the appropriate icon and it will bring up the window to look for your sequence straight away. To print a melting profile, just click on the printer icon and the print preview will come up. You can also save a sequence or method in the same way.
19. A melting profile produced in the top graphic: *helical fraction vs temperature* that has an S-shaped curve melting over 4–6 degrees is likely to have one major melting domain or multiple domains close together (**Fig. 3**). Melting over a wider range or a differently shaped profile suggests the presence of multiple distinct melting domains (**Fig. 4**).

The bottom graphic (**Figs. 3, 4**) shows the calculated  $T_m$  of each individual base-pair (bp) in the sequence plotted against bp position. This can also be viewed with bp position plotted on the Y axis and temperature on the X axis to check the melting temperature of the major domains. If only one domain is present, analysis at the  $T_m$  will usually be appropriate. If more than one domain exists, but these are separated by no more than 3°C, a temperature at or close to the mean  $T_m$  will usually suffice. Domains more than 3°C apart may require analysis at more than one temperature when screening for unknown mutations, and these temperatures can be derived from the graphic. For fragments with one domain or domains less than 3°C apart, select the “trial function” and enter values around the  $T_m$  (e.g.,  $\pm 1^\circ\text{C}$ ) and press “calculate.” For fragments with more than one domain, start at 1°C lower than the lowest melting domain and evaluate the helical fraction across the fragment at progressively higher temperatures. Although a helical fraction of 75% is ideal, this will rarely be maintained along the whole fragment but a temperature associated with a helical fraction of 60–90% should be identified. Large fragments (>500 bp) are likely to have more than one melting domain, and it may be best to redesign primers or cut the fragment with a restriction enzyme.

If more than one temperature is used, we usually choose  $>1^\circ\text{C}$  intervals, using as few temperatures as possible. The melting temperature of the ends of the fragment are irrelevant, as the first 20 bases are primer. Mutations near the primers are usually seen easily, since they will be into the first major domain. When a small part of a fragment has a significantly higher melt domain than the remainder, it is advisable to select a temperature that is 1°C lower than the predicted temperature for that domain. If the running temperature differs from  $T_m$ , a time shift of generally  $\pm 0.5$  min per  $1^\circ\text{C}$  will be required to elute the fragment at the correct time.

It is worth noting that melting profiles prepared with WAVEmaker 4.0 or above may differ from those prepared for the same fragment with WAVEmaker 3.4. Versions 4.0 and above are recommended.

## Acknowledgments

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## Mutation Scanning for the Clinical Laboratory—Protein Truncation Test

Yvonne Wallis

### 1. Introduction

The protein truncation test (PTT) is a powerful mutation detection technique originally described for mutation screening of the dystrophin gene (*D*). It is based on in vitro transcription/translation technology, and is capable of directly scanning kilobase-sized fragments of DNA for chain-terminating mutations. PTT should be the mutation detection technique of choice to screen genes in which the majority (if not all) of the disease-causing mutations result in the formation of a truncated protein product.

During the PTT, polymerase chain reaction (PCR)-amplified coding sequences are transcribed and then translated in vitro to produce proteins. The presence of a chain-terminating mutation in the PCR product will result in premature termination of translation, thus leading to the synthesis of a truncated protein product. PCR templates for the PTT reaction are generated from either genomic DNA or, more often, from cDNA obtained through the reverse transcription-PCR (RT-PCR) of mRNA (*see Note 1*).

The choice of starting material (genomic DNA or RNA) is determined by the genomic structure of the gene to be screened. RNA is required to screen genes with many small exons—e.g., the dystrophin gene—whereas genomic DNA is the preferred option for genes with very large stretches of coding sequence, such as the *APC* and *BRCA* genes.

The success of the PTT relies upon the incorporation of a nonspecific 5' tail during the PCR amplification stage (*see Note 2*). The 5' tail contains components that are essential for in vitro transcription and subsequent translation of the PCR product, and contains four key regions (*2*). A T7 promoter sequence

at the most 5' end directs the production of RNA, and is separated from a eukaryotic translation initiation or Kozak sequence (3) by a spacer of 3–6 bp. The 3' end of the tail contains an in-frame ATG codon followed by the gene-specific target sequence. The sequences of the four regions of the 5' tail are based on the following:

<u>T7 promoter sequence</u>	<u>Spacer</u>	<u>Kozak</u>	<u>In-frame initiation codon</u>
TAATACGACTCACTATAGG	AACAG	CCACC	ATG . . (gene-specific sequence)

Transcription and translation reactions may be conveniently performed in a single-tube assay using the TNT<sup>®</sup> T7 Quick Coupled Transcription/Translation system (Promega, Madison, WI). This system uses a TNT<sup>®</sup> Quick Master Mix, which contains the RNA polymerase, nucleotides, salts, Recombinant RNasin<sup>®</sup> Ribonuclease Inhibitor and rabbit reticulocyte lysate solution.

The PTT offers a number of advantages over other mutation detection techniques, including:

1. Large coding segments (2–3 kb) can be screened in a single assay (*see Note 3*). Fewer PCR fragments are therefore required to analyze a gene using this technique, compared to other more commonly used methods such as SSCP and DGGE. For example, 35 PCR fragments are required to screen the *APC* gene by DGGE, whereas just five are required using the PTT.
2. As this technique detects chain-terminating mutations, time and effort are saved by not sequencing non-pathogenic variants picked up by other techniques.
3. The size of the truncated product will predict the approximate position of the DNA sequence alteration. This is helpful when subsequent sequencing analysis is required to characterize the exact nature of the chain-terminating mutation.

There are also a number of limitations, including:

1. Missense mutations are not usually detected by the PTT (*see Note 4*). Thus, it would not be an appropriate technique to use for those conditions in which amino acid changes are often pathogenic.
2. Like most other routine mutation detection techniques, PTT will not detect large gene deletions/gross rearrangements (unless RT-PTT is performed).
3. The requirement for RNA may be a limiting factor. Most diagnostic laboratories are set up for DNA-based screening techniques, and will therefore require a second fresh blood sample for RNA extraction to perform RT-PTT reactions.
4. The extraction of high-quality RNA is essential for PTT, and therefore, care must be taken to avoid degradation by RNA nucleases.

The PTT can be divided into four stages (*see Fig. 1*):

Stage 1: PCR amplification of the starting template, either genomic DNA or cDNA derived from RT-PCR of mRNA;

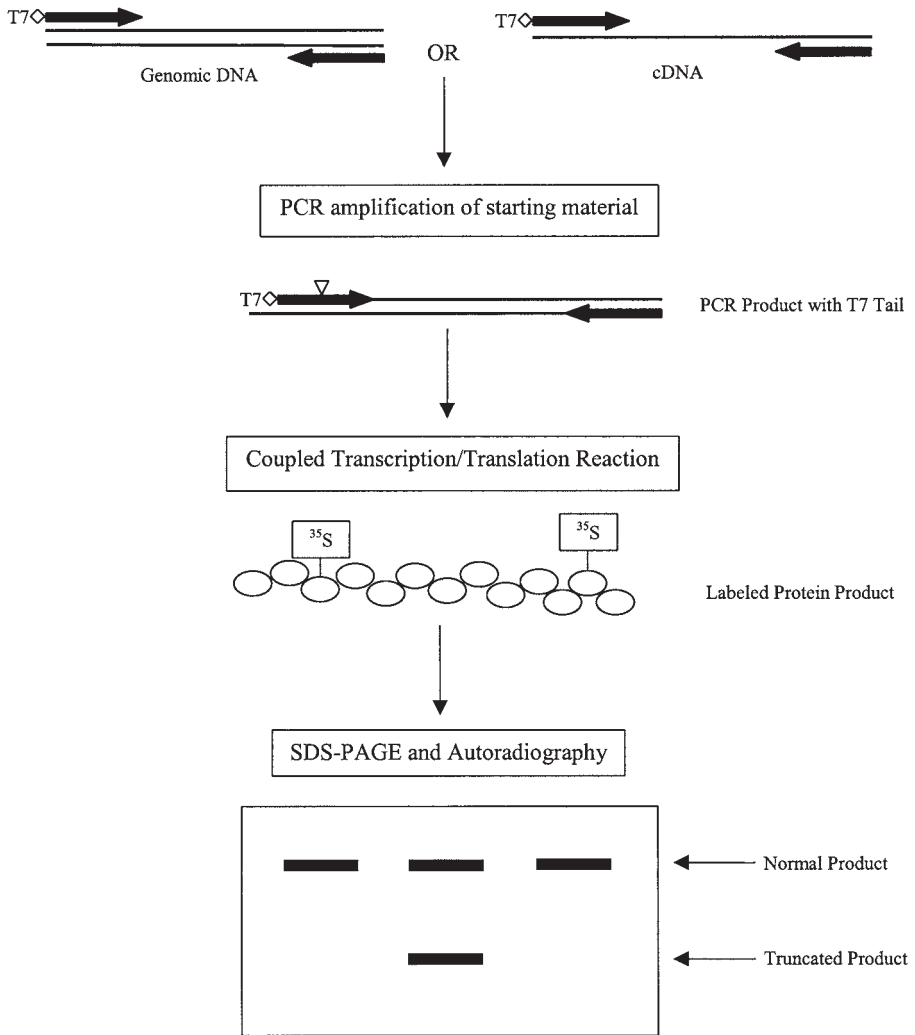


Fig. 1. Schematic diagram representing the four key stages of the protein truncation test: PCR; coupled transcription/translation; SDS-PAGE; and autoradiography. The “T7” and diamond shape represent the T7 promoter sequence and Kozak translation initiation site, respectively.

Stage 2: *in vitro* transcription and translation of PCR products to produce radiolabeled proteins. [ $^{35}\text{S}$ ]methionine is the radiolabel of choice, but others may be used, such as [ $^{14}\text{C}$ ]leucine and [ $^3\text{H}$ ]leucine, if there is a scarcity of methionine residues at the 5' end of the protein product (leucine is one of the most frequently represented amino acids).

Non-isotopic detection systems are now available if radioactive protocols are prohibitive (*see Note 5*);

Stage 3: sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to resolve the protein products;

Stage 4: visualization of the labeled protein products—e.g., by autoradiography for radiolabeled proteins.

The following Materials and Methods are based upon standard protocols recommended by Promega (*see* Technical Manual No. 045 supplied with TNT<sup>®</sup> Quick Coupled Transcription/Translation Systems kit) and are used to screen exon 15 of the *APC* gene in the author's laboratory. Modifications may be necessary for successful PTT analysis of other genes. **Table 1** describes a number of gene-specific modifications obtained from recent literature, and may therefore be a useful point of reference.

## 2. Materials

### 2.1. Reverse Transcription of RNA

All components are supplied by Promega, Madison, WI unless otherwise stated.

1. Random hexamers: 0.5  $\mu\text{g}/\mu\text{L}$ .
2. M-MLV reverse transcriptase, RNase H minus: 100–200 U/ $\mu\text{L}$ .
3. RNasin ribonuclease inhibitor: 20–40 U/ $\mu\text{L}$ , store at  $-20^{\circ}\text{C}$ .
4. 5X reverse transcriptase reaction buffer: 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM  $\text{MgCl}_2$ , 50 mM dithiothreitol (DTT). Store at  $-20^{\circ}\text{C}$ .
5. Deoxynucleotide 5' triphosphate (dNTP) Mix: each dNTP is supplied at 100 mM stock concentration. Prepare a 2-mM working stock mix containing all four dNTPs and store at  $-20^{\circ}\text{C}$ .
6. DTT: 0.1 M solution, store at  $-20^{\circ}\text{C}$ .
7. RNase-free water.

### 2.2. Polymerase Chain Reaction (PCR)

All PCR components are supplied by Invitrogen Life Technologies, Paisley, UK unless otherwise stated.

1. *Taq* DNA polymerase: 5 U/ $\mu\text{L}$ , store at  $-20^{\circ}\text{C}$ .
2. 10X *Taq* DNA polymerase buffer: 200 mM Tris-HCl (pH 8.4), 500 mM KCl, store at  $-20^{\circ}\text{C}$ .
3. dNTP mix: each dNTP is supplied at 100 mM stock concentration. Prepare a 2 mM working stock mix containing all four dNTPs and store at  $-20^{\circ}\text{C}$ .
4. Oligonucleotide primers (Cruachem Ltd, Northumberland, UK): Supplied lyophilized. All stock primers are made to a final concentration of 250  $\mu\text{M}$  with water. Working primers are diluted to 25  $\mu\text{M}$ .



**Table 1**  
**Examples of Gene-Specific Modifications Made**  
**to the Standard PTT Protocol Described in the Text**

Disease	Gene	Starting material	Disease-specific comments (refs.)
Duchenne muscular dystrophy	<i>DMD</i>	RNA	Multiplex PTT reaction of five RT-PCR fragments covering 11-kb coding region ( <b>9</b> )
Hereditary Breast/ovarian cancer	<i>BRCA1</i> <i>BRCA2</i>	RNA, DNA	Two-round PCR amplification of cDNA. 50 cycle PCR for genomic DNA. Use of cycloheximide to reduce non-sense-mediated mRNA decay ( <b>5</b> )
Neurofibromatosis type I	<i>NF1</i>	RNA	Expand high-fidelity PCR system used to amplify cDNA templates ( <b>10</b> ).
Stickler syndrome	<i>COL2A1</i>	RNA	Lymphoblasts and fibroblasts pre-incubated with cycloheximide to reduce non-sense-mediated mRNA decay ( <b>11</b> )
Cystic fibrosis	<i>CFTR</i>	RNA	Two-round PCR amplification of cDNA ( <b>12</b> )
Autosomal dominant polycystic kidney disease	<i>PKD1</i> , <i>PKD2</i>	RNA	Taq Extender used to amplify cDNA template in a DMSO containing buffer ( <b>13</b> )
Tuberous sclerosis	<i>TSC1</i> , <i>TSC2</i>	RNA	Touchdown PCR amplification of cDNA. SDS PAGE gels soaked in 1 M sodium salicylate before drying ( <b>14</b> )

5. Magnesium chloride: 25 mM, store at  $-20^{\circ}\text{C}$ .
6. Water: injection grade (Braun Medical Ltd., UK).
7. If necessary, mineral oil (Sigma-Aldrich, St. Louis, MO): store at room temperature.
8. Agarose: 1% (w/v) solution in 1X TBE containing 400  $\mu\text{g/L}$  of ethidium bromide.
9. Electrophoresis TBE buffer (Severn Biotech Ltd., UK): supplied as a 10X stock. Diluted to a 1X working solution with distilled water.
10. Agarose electrophoresis equipment.

### **2.3. PTT: In Vitro Transcription/Translation of PCR Product**

All components are supplied in the TNT<sup>®</sup> T7 Quick Coupled Transcription/Translation System (Promega) unless otherwise stated. All components of the

kit are stored at  $-70^{\circ}\text{C}$ . Unfoiled Lysate should never be stored in the presence of dry ice.

1. TNT<sup>®</sup> T7 Quick Master Mix: stored at  $-70^{\circ}\text{C}$ . Do not freeze-thaw the master mix more than twice, as this may result in loss of translational activity.
2. [<sup>35</sup>S]methionine: supplied by Amersham Pharmacia Biotech, Bucks, UK (1000 Ci/mmol at 10 mCi/mL). Store at  $-20^{\circ}\text{C}$  (*see Note 6*).
3. T7 TNT<sup>®</sup> PCR Enhancer (*see Note 7*).
4. Nuclease-free water.
5. 2X SDS-PAGE sample loading buffer: 100 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, 0.05% (w/v) bromophenol blue. Store at room temperature.

#### **2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of PTT Products**

1. PAGE unit: e.g., MiniProtean II gel system (Bio-Rad Laboratories Richmond, CA).
2. 40% stock polyacrylamide solution (Sigma-Aldrich): 29:1 acrylamide: bisacrylamide. Store at  $4^{\circ}\text{C}$ .
3. Tris buffer (for resolving gel): 1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8, store at  $4^{\circ}\text{C}$ .
4. Tris buffer (for stacking gel): 0.5 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8, store at  $4^{\circ}\text{C}$ .
5. 10% SDS: ready-made solution (Invitrogen Life Technologies).
6. Ammonium persulfate solution (APS): 10% (w/v). Can be stored in 500  $\mu\text{L}$  aliquots at  $-20^{\circ}\text{C}$ . Do not freeze-thaw more than once.
7. TEMED: store at room temperature.
8. 0.1% SDS solution: store at room temperature.
9. 1X SDS-PAGE running buffer: made up as a 10X solution and diluted 1:10 just before use. Store at  $4^{\circ}\text{C}$ . 1X solution consists of 25 mM Tris base (3.0 g/L), 192 mM glycine (14.4 g/L) and 0.1% (w/v) SDS (10 mL of 10% SDS/L).
10. SDS-PAGE gel fix solution: aqueous solution of 7% (v/v) acetic acid. A methanol/acetic acid fixing solution may also be used, consisting of an aqueous solution of 10% (v/v) methanol and 10% (v/v) acetic acid.
11. Whatman<sup>®</sup> 3MM paper (Whatman, Maidstone, UK).
12. Vacuum slab gel drier.
13. Saran Wrap: or any similar cling film.
14. X-ray film: e.g., Kodak X-Omat AR (Kodak, Cambridge, UK).

### **3. Methods**

#### **3.1. Reverse Transcription of RNA**

This procedure is necessary when starting from RNA, as it provides a cDNA template for subsequent PCR. All care must be taken to avoid contamination with RNases (*see Note 1*).

1. Add together the following components: 2  $\mu\text{L}$  random hexamer and 3  $\mu\text{g}$  of total RNA. Make up to a final volume of 30  $\mu\text{L}$  with TE. Incubate the mix at 65°C for 10 min and place immediately on ice.
2. To the RNA/primer mix, add directly (or make a pre-mix if preferred) the following components and incubate at 37°C for 60 min:
  - a. 12  $\mu\text{L}$  5X reverse transcriptase reaction buffer.
  - b. 1  $\mu\text{L}$  RNasin, 6  $\mu\text{L}$  dNTP mix.
  - c. 6  $\mu\text{L}$  DTT, 3  $\mu\text{L}$  M-MLV reverse transcriptase.
  - d. 2  $\mu\text{L}$  sterile water to a final vol of 60  $\mu\text{L}$ .
3. Use immediately in PCR or store at -20°C until needed.

### 3.2. PCR

When using RT-PCR, it may be necessary to perform a two-stage amplification reaction to amplify the target sequence, in the cDNA product, to a detectable level (e.g., for DMD testing). In stage one, cDNA is used as the starting material (e.g., 10  $\mu\text{L}$  of a 60  $\mu\text{L}$  RT reaction volume). A small aliquot of the stage-one PCR product (e.g., 3  $\mu\text{L}$  from a 50  $\mu\text{L}$  reaction volume) is then used as the starting material for stage two. The stage two PCR product can then be used for the PTT reaction. The stage two forward primer is modified, e.g., it contains the T7 RNA polymerase promoter sequence, a translation initiation sequence and an in-frame ATG codon.

Amplification of genomic DNA, in contrast, requires just a single stage of amplification, using 200–500 ng of template.

The following PCR protocol is based on that used to amplify segments 2–5 of exon 15 of the *APC* gene. Adjustment of gene-specific components, and stages will therefore be required for screening other genes.

A sample from a known normal individual should always be included as well as a “no DNA” negative control. It is also worth running known mutant controls along with the screening samples to facilitate sizing of any “new” unknown truncations.

1. To each 0.6 mL tube, add 1  $\mu\text{L}$  genomic DNA (equivalent to 200–500 ng of DNA). If amplifying a cDNA template, add 10  $\mu\text{L}$  of cDNA.
2. Prepare a master mix to yield final concentrations of the following components:
  - a. 1X PCR buffer.
  - b. 1.5 mM  $\text{MgCl}_2$ .
  - c. 200  $\mu\text{M}$  dNTP.
  - d. 25  $\mu\text{M}$  of forward and reverse gene-specific primers.
  - e. 1 U *Taq* DNA polymerase.
  - f. Water up to a final volume of 25  $\mu\text{L}$ .If a hot start is required, omit the *Taq* DNA polymerase.
3. Add an appropriate volume of the PCR master mix to each tube containing the DNA template, and if necessary overlay each reaction with 20  $\mu\text{L}$  of mineral oil.

Place the tubes into a thermal cycler to perform the PCR. Cycling conditions are gene-specific, but usually include an initial denaturation step at 95°C for at least 3 min, followed by 35 cycles of denaturation (95°C for 1 min), annealing (at the annealing temperature for 1 min) and extension (72°C for 1–2 min, depending on the size of the amplicon), followed by a final extension at 72°C for 10 min. A high yield of PCR product is essential for the subsequent PTT reaction; therefore, perform at least 35 cycles of PCR.

4. If a hot start is required, add the *Taq* DNA polymerase (usually 1  $\mu$ L of enzyme diluted 1:5 with water) to each reaction tube during the initial denaturation step.
5. When cycling is complete, analyze the PCR products (5  $\mu$ L of each PCR plus 2  $\mu$ L loading buffer) by agarose gel electrophoresis to check the specificity and yield of PCR product (see **Note 8**). Abnormally sized products generated by RT-PCR indicate the presence of mutations affecting RNA splicing or genetic rearrangements (e.g., caused by deletions or duplications). 1% agarose gels are used to analyze PCR products of 1–3 kb in size.

### **3.3. PTT: In Vitro Transcription/Translation of PCR Product**

It is not necessary to purify the PCR products before they go into the coupled in vitro transcription-translation reaction.

1. Rapidly thaw the TNT<sup>®</sup> T7 Quick Master Mix by hand-warming and then place on ice.
2. In a 0.6 mL tube containing 1–2.5  $\mu$ L PCR-generated template (depending on the yield of PCR product), add the following components (or prepare a master mix if preferred):
  - a. 0.25  $\mu$ L T7 TNT<sup>®</sup> PCR Enhancer.
  - b. 0.5  $\mu$ L [<sup>35</sup>S] methionine.
  - c. 9.5  $\mu$ L TNT<sup>®</sup> T7 Quick Master Mix.
  - d. Nuclease-free water up to a final vol of 12.5  $\mu$ L.This is one-quarter of the total volume suggested by Promega, but works perfectly well.
3. Incubate the reaction at 30°C for 90 min (see **Note 9**). The reactions may then be stored at –20°C until needed.
4. A negative control should also be included in the PTT reaction (e.g., a sample that does not contain any PCR product), as this reaction will show any background incorporation of labeled amino acid.
5. Add 50  $\mu$ L SDS-PAGE sample buffer and denature the protein products at 70°C for 10 min (see **Notes 10** and **11**).

### **3.4. SDS-PAGE Analysis of PTT Products**

The protein products are subsequently resolved by SDS-PAGE. The MiniProtean II gel assembly (BioRad Ca) analyzes gels that have final dimensions of 10  $\times$  10  $\times$  0.8 cm, and can therefore be prepared while the PTT reactions are incubating.

1. Prepare two 15% SDS-PAGE (the resolving gels, *see Note 12*) for each set of 20 samples (each gel will hold 10 samples) from the following components:
  - a. 3.75 mL 40% polyacrylamide stock.
  - b. 2.5 mL 1.5 M Tris at pH 8.8.
  - c. 100  $\mu$ L 10% SDS.
  - d. 3.7 mL distilled water (up to a final vol of 10 mL).
  - e. 50  $\mu$ L 10% APS.
  - f. 5  $\mu$ L TEMED.
2. Overlay each resolving gel with 100  $\mu$ L 0.1% SDS (produces a level gel surface) and leave to polymerize (for at least 30 min). The 0.1% SDS layer is removed immediately before pouring the stacking gel.
3. Prepare 5% stacking gels from the following components:
  - a. 0.625 mL 40% polyacrylamide stock.
  - b. 0.625 mL 1.0 M Tris at pH 6.8.
  - c. 50  $\mu$ L 10% SDS.
  - d. 3.6 mL distilled water (up to a final vol of 5 mL).
  - e. 50  $\mu$ L 10% APS.
  - f. 5  $\mu$ L TEMED.Leave to polymerize (for at least 15 min).
4. Remove the combs and assemble the gel apparatus. Flush the wells with 1X running buffer.
5. Load 15  $\mu$ L of each denatured sample. Allow frozen samples to thaw thoroughly before loading (*see Note 13*). If required, load appropriate protein mol-wt markers for subsequent sizing of protein products (*see Note 14*). Markers are available from Bio-Rad Laboratories (Richmond, CA).
6. Run at constant current (30 mA for each gel) until the bromophenol blue reaches the bottom of the gel. It takes approx 90 min for the bromophenol blue to reach the bottom of the Bio-Rad Mini-Protean II gels running at 30 mA per gel. Running time will differ for other systems.
7. Fix the gels in fixing solution for 30 min, with gentle agitation on an orbital shaker (*see Note 15*).
8. Rinse the gels in distilled water for 5–10 min (*see Note 15*).
9. Transfer the gels onto Whatman<sup>®</sup> 3MM paper and cover with plastic wrap. Dry the gel completely using a heated vacuum dryer. The 10  $\times$  10  $\times$  0.8 cm Bio-Rad gels take 50–60 min to dry at 60°C. Drying times and temperatures for alternatively sized gels should be adjusted accordingly.
10. Place the gels in to an X-ray film cassette with a sheet of X-ray film and leave overnight at room temperature (*see Note 17*).
11. Develop the film the next day. Any truncated protein products will migrate faster than full-length peptides and should be easily visible (**Fig. 2**).
12. Unexpected bands on the gel may make interpretation more difficult. Alternately, spliced forms can produce extra bands in RT-PCR-based PTT. However, there are a number of other potential explanations related to the in vitro transcription-translation process itself (*see Note 18*).

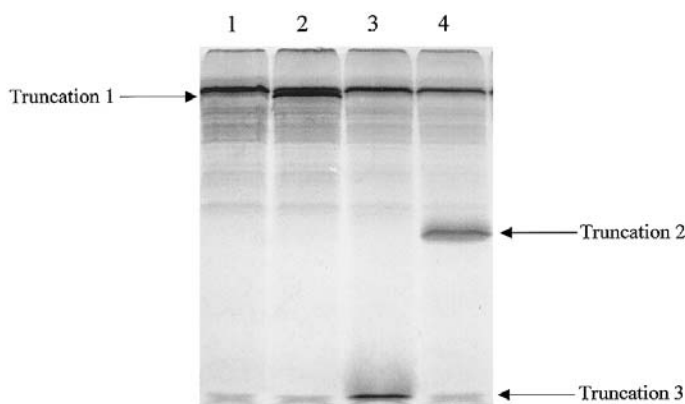


Fig. 2. PTT analysis of segment 2 of exon 15 of the APC gene (codons 654 to 1263). Lane 1: normal control. Lane 2: truncation 1 caused by a C to A base substitution in codon 1201. Lane 3: truncation caused by a 1 bp deletion from codon 728. Lane 4: truncation 3 caused by a 1 bp insertion in to codon 831.

#### 4. Notes

1. The quality of the RNA is very important in the RT-PCR-based PTT process. The best source of RNA is cells in which the target gene is abundantly expressed. However, for practical reasons, RNA is generally isolated from freshly drawn peripheral-blood lymphocytes or cultured skin cells. It is important to prevent contamination by RNases during the extraction procedure. Gloves (changed frequently) should always be worn, and solutions should be DEPC-treated.
2. If expression of the full-length protein product (e.g., wild-type fragment) is difficult, it may be worth adding a stop codon at the end of the PCR product, best achieved by incorporating it at the end of the downstream primer. The stop codon prevents ribosomes from stalling at the ends of linear DNA. Increased expression may also be achieved by using PCR templates that contain a poly(A) sequence downstream of the coding sequence. Again, this can be incorporated at the end of the downstream primer. Poly(A) sequences are important for mRNA stability, and can play a role in translation initiation in Rabbit Reticulocyte Lysate (4).
3. PCR products longer than ~3300 bp are less efficiently transcribed/translated than smaller products (5). Very large coding regions should therefore be divided into overlapping segments for PCR and subsequent PTT analysis. In addition, it is important to consider the amount of overlap between two overlapping PTT fragments. Generally, 130 amino acids is considered the lower limit of detection for PTT using 10–15% SDS-PAGE gels in conjunction with a tris/glycine buffer system (6).
4. A missense mutation caused by an A to T base substitution in exon 15 of the APC gene (I1307K) is detectable by the PTT (7). This mutation is present in up to 6% of the Ashkenazi Jewish population, but does not segregate with FAP itself. The

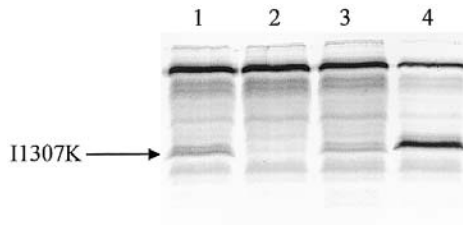


Fig. 3. PTT analysis of segment 3 of exon 15 of the APC gene (codons 990 to 1700). Lane 2: normal control. Lanes 1 and 3: truncations caused by 11307K missense mutation. Lane 4: truncation caused by a 5-bp deletion from codon 1309.

poly(A)<sub>8</sub>) tract created by this base substitution is thought to result in DNA replication errors (at least *in vitro*), leading to a frameshift mutation and thus a downstream stop codon (*see Fig. 3*).

5. Two non-isotopic detection systems are available from Promega, the Transcend™ System (*see* Technical Manual No. 045 supplied with TNT® Quick Coupled Transcription/Translation Systems kit) and the FluoroTect™ Green Protein Labeling System (8). The Transcend™ system incorporates biotinylated lysine residues into the protein products during translation. The drawback with this system is that the proteins must then be electroblotted to a solid matrix (e.g., PVDF membrane) so that the biotinylated proteins can be detected nonisotopically using streptavidin-alkaline phosphatase or horseradish peroxidase conjugates and either colorimetric or chemiluminescent detection reagents. The FluoroTect™ Green system uses a charged lysine tRNA molecule labeled with the BODIPY®-FL fluorophore added to the translation reaction. Following SDS-PAGE, the labeled proteins can be immediately detected directly “in-gel” by use of a laser-based fluorescent gel scanner (most likely the limiting factor for diagnostic laboratories).
6. Promega recommend using a grade of [<sup>35</sup>S]methionine, such as Amersham International Redivue™ L-[<sup>35</sup>S]methionine, which does not cause background labeling of the 42-kDa rabbit reticulocyte protein present in the master mix. Background labeling would produce extra bands on the gel.
7. The T7 TNT® PCR Enhancer contains magnesium acetate and potassium chloride, which increase both the efficiency and fidelity of the transcription/translation reaction.
8. The best PTT results are obtained with high yield, specific PCRs. Mis-priming leads to the synthesis of extra nonspecific proteins, which will produce a high background, making interpretation more difficult. It is worth spending time optimizing the PCR, or if necessary, redesigning primers which produce a “cleaner” product.
9. The optimal coupled transcription/translation reaction occurs in 60–90 min at 30°C. Incubation at 37°C will reduce the efficiency of translation.
10. SDS sample buffer may be added prior to freezing if the samples are not needed immediately after the transcription/translation reaction. The samples may be dena-



tured prior to storage at  $-20^{\circ}\text{C}$ . The samples do not need to be denatured again prior to electrophoresis.

11. It is not necessary to denature the protein samples at  $95^{\circ}\text{C}$ . Denaturation at this temperature may result in unexpected bands at higher mol wts.
12. The MiniProtean II gel electrophoresis unit is relatively small. If better resolution of higher mol-wt proteins is required (produced as a result of chain-terminating mutations toward the 3' end of the PCR product), PCR products should also be run on a 10% gel. A single 12% gel may be used on larger gel systems if preferred (such as the 20 cm  $\times$  20 cm  $\times$  0.1 cm Bio-Rad Protean II system). Pre-cast gradient gels may also be used (e.g., the 4–20% gradient gels from NOVEX™ and Bio-Rad), which facilitate the resolution of proteins over a wide range of mol-wt sizes.
13. Too much protein loaded on to the gel may cause smearing. Therefore, try to reduce the volume of sample loaded if smearing is a problem.
14. It is possible to estimate the size of the truncated protein products using a protein size marker. However, it should be remembered that the actual position of the chain-terminating mutation may be some distance upstream of the actual stop codon. It is therefore very important to run known mutant samples to gauge the most appropriate sequencing reaction to use for subsequent characterization of the mutation.
15. If gel cracking becomes a problem, it may be worth soaking the gel in a solution of 7% acetic acid/7% methanol/1% glycerol for 5 min prior to drying.
16. Washing the gel in water for 5–10 min before drying helps to prevent gel smearing.
17. Labeled protein bands in gels may also be visualized by fluorography, a method that increases the sensitivity of detection of  $^{35}\text{S}$ -,  $^{14}\text{C}$ -, and  $^3\text{H}$ -labeled proteins. The increased sensitivity is achieved by infusing an organic scintillant (e.g., Amplify™ Reagent from Amersham) into the gel, which converts the emitted energy of the isotope to visible light and therefore increases the proportion of energy that may be detected by X-ray film.
18. The presence of unexpected bands on a gel may be the result of a number of different causes. More than one peptide may be translated from the template as a result of translation initiation at internal methionines. Optimization of the magnesium and potassium concentrations may increase fidelity (e.g., addition of the Enhancer). Background bands may also be caused by the presence of globin (migrates at 10–15 kDa) or aminoacyl tRNAs (migrate at approx 25 kDa). Both proteins are components of the rabbit reticulocyte lysate.

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## Mutation Scanning for the Clinical Laboratory

### *Automated Fluorescent Sequencing*

**Andrew J. Wallace**

#### **1. Introduction**

Automated fluorescent sequencing is often overlooked as a point mutation scanning technique usually on the grounds of cost, technical complexity, and difficulties in processing the information generated. However, sequencing cannot be avoided completely, because regardless of the mutation scanning technique employed, it must ultimately be used for mutation characterization.

Since any active mutation scanning laboratory will have to contemplate sequencing to define variants, it is worth taking a second look at sequencing's benefits compared to other technologies. Sequencing is highly sensitive (approaching 100% for point mutations), and it fully characterizes point mutations in one step. Sequencing as a primary mutation scan also permits organizational simplicity in the laboratory, because there is no need to operate and troubleshoot a pre-screen technique. A further advantage is that the technique has benefited from the huge commercial and academic investments that have resulted from genome-sequencing projects. This investment is likely to continue in the future, leading to further improvements and efficiencies.

The process of fluorescent sequencing is multi-stage. First, the template DNA is prepared. In clinical laboratories, this is done almost exclusively by polymerase chain reaction (PCR) amplification. Second, the template DNA is purified free from PCR primers, unincorporated nucleotides and the PCR buffer. The template is now ready for DNA sequencing. Once sequencing is complete, unincorporated dye-labeled fluorescent dideoxyterminators must be removed.

The purified sequencing products are then subject to high-resolution electrophoresis on a fluorescent sequencer. Finally, the electronic output, or electropherogram, from the sequencer is analyzed for the presence of mutations—preferably using a software package, or alternatively by eye.

### **1.1. Fluorescent Sequencing Platforms**

Fluorescent sequencers are available from several manufacturers. Applied Biosystems (ABI) are currently the market leaders, although Pharmacia, Beckman, and Li-Cor offer serious competition. Traditionally, automated sequencers have relied on slab gel electrophoresis. However, an important change has occurred within the last few years as sequencers have entered the market that carry out electrophoresis in capillaries using flowable polymers as a separation matrix—e.g., ABI 3700, 3100, 310, Pharmacia MEGABace, and Beckman CEQ 2000XL. Their introduction has considerably simplified the process of automated sequencing as gel pouring and sample loading have been eliminated. Nevertheless, some currently available sequencers remain reliant on traditional slab polyacrylamide gels, such as ABI 377, and Li-Cor. At the present time, capillary-based sequencers offer distinct advantages with respect to automation and hands-on running time, whereas slab gel sequencers still offer superior read lengths and overall sequence quality when they are operated rigorously to a high standard. Nevertheless, 80-cm capillary arrays have become available for the ABI 3100. The sequencing read lengths generated using these are comparable to those achieved with the ABI 377 long-read protocol (*see Subheading 3.4.*) and may become superior in the future.

The protocols and methods provided in this chapter relate principally to the operation of ABI fluorescent sequencers, because the author's experience is limited to the ABI 373, 377 and 3100. Hopefully, there will be many protocols that will be useful to users of other instruments.

### **1.2. Sequencing Chemistries**

Sequencing chemistry must be considered when carrying out mutation scanning. The chemistry is to some extent determined by the sequencing platform available in the laboratory. The Li-Cor and Beckman CEQ 2000XL are both tied to their own sequencing chemistries, whereas there is compatibility between the chemistries employed by the Pharmacia MEGABace and ABI instruments (377, 3100, 3700). The second consideration is the choice between dye-labeled primer and dye-labeled terminator chemistries. The alternatives will be partly determined by particular advantages of the chemistries available for the chosen platform. Dye-labeled primer chemistries are generally superior for the purposes of heterozygote detection because they give less variability between peak incorporations than dye terminators. However, dye primer chemistries are more

time-consuming to set up because there are four separate termination reactions per sample (effectively two with the bi-directional approach used on the Li-Cor). Furthermore, there is the disadvantage that dye primer chemistries are prone to pausing artefacts which can obscure portions of the read, whereas dye terminator chemistries are not susceptible to this problem. Dye terminator chemistries have the advantage that they are more simple to set up because most permit a single sequencing reaction to be carried out within a single tube. Dye terminator chemistries have also undergone substantial improvements with respect to both engineered sequencing enzymes and improved dye labels. The DYEnamic ET and BigDye™ Terminator Chemistries (v2.0 and v3.0) are both extremely effective, and the general consensus is that their convenience outweighs the marginal improvement in incorporation evenness offered by the corresponding dye primer chemistry.

### **1.3. Template Preparation**

This is a critical stage. The quality of sequence data generated is heavily dependent on the quality of template used. A good-quality template consists of a single specific DNA fragment, at high copy number, with minimum carry over of the components of the original PCR amplification—e.g., unincorporated deoxynucleotide 5′triphosphates (dNTPs) and primers. Time is well spent on optimizing this step, especially when a fragment will be regularly and repeatedly sequenced as part of a mutation scanning service. Careful primer design and the use of “hot start” PCR can drastically improve the specificity and efficiency of the PCR reaction. A multitude of protocols are available for PCR “clean up” prior to sequencing, each with its own advantages and disadvantages. The main variables to consider when choosing a protocol are cost, efficiency, ease of use, and suitability for high throughput.

### **1.4. The Sequencing Primer**

The design of the sequencing primer is another important factor that determines the quality of sequence data achieved. Factors to consider are the melting temperature ( $T_m$ ) of the primer, which ideally should be within the range of 55–60°C, and the sequence composition, which should be about 50% GC content. The quality of primer synthesis is also very important. Poor primer syntheses and crude primer preparations contain truncated oligonucleotide fragments. Once at or above a significant level (>5%), their presence leads to increased background noise and degrades the quality of sequence. Primers used for sequencing are often identical to the primers used for the primary PCR; however, they do not need to be. Cleaner sequencing reactions can be obtained by using an internal nested sequencing primer. Alternatively, the PCR primers can incorporate “universal” tails at their 5′ end, thus permitting sequencing using

“universal” sequencing primers. Provided that the universal tails are synthesized on primers consistently, this strategy can be employed to facilitate high-throughput sequencing, as it permits batch preparation of unrelated sequencing reactions. One useful secondary effect is that a higher proportion of the fragment being sequenced is within the good-quality read, as the first 15–20 nucleotides of the sequencing ladder comprise the specific portion of the PCR primer.

### **1.5. Principle of Fluorescent Cycle Sequencing**

Fluorescent cycle sequencing is a derivation of the dideoxy chain termination method first developed by Sanger et al. (1). The main modifications to the original method are the use of a thermostable polymerase, permitting repeated cycles of annealing, and extension/termination of the sequencing primer on the template, and the substitution of fluorescent dye labels—either on the sequencing primer or the terminator—rather than radioisotope labels. These modifications confer the advantage of greater sensitivity—less target DNA must be prepared relative to single extension protocols—and greater robustness, as the high extension temperatures possible with thermophilic sequencing enzymes reduces the potential for secondary structure formation on the template DNA. Cycle sequencing is a form of PCR. Like PCR, a cycle sequencing reaction undergoes successive cycles of denaturation, annealing, and extension/termination. However, the presence of only one primer means that the products accumulate linearly rather than exponentially.

### **1.6. Unincorporated Dye Terminator Removal**

When using dye-labeled terminator chemistries, the unincorporated terminators must be removed from the sequencing reaction prior to gel electrophoresis. Failure to remove them efficiently leads to part of the sequence data being obscured (particularly the sequence close to the primer) and thus loss of data. Many protocols and proprietary kits are available to remove dye terminators. The main considerations are cost, consistency, ease of use, and suitability for high throughput.

### **1.7. Data Analysis**

Data analysis must be fast and effective when using sequencing as a mutation scan. Large amounts of information are generated by each sequencing reaction, and organizing this data effectively and rapidly for heterozygote bases poses problems. There are many software packages on the market that are capable of this. Most of these operate on the principle of comparing the peak height of the called base with the peak heights of uncalled subsidiary peaks. The operator sets a threshold that they are willing to tolerate as a percentage of the called base height and the software flags up those bases that exceed the pre-set thresh-



old. The heterozygote detection component of Sequencher (Genecodes Corporation) operates on this principle as well as Factura and SeqScape (both from Applied Biosystems [ABI]). An alternative approach is to use trace subtraction. Here, the test (patient) sequence is compared with a reference (control) sequence by mathematically subtracting one from the other. The resultant “difference” trace can be inspected, highlighting any differences between the two sequences (2). The Gap4 database viewer (available as part of the Staden package of sequence analysis programs) has the ability to carry out trace subtraction. Another alternative is the Comparative Sequence Analysis (CSA) approach (3), which uses the fragment analysis program Genescan (ABI) to split the sequencing reaction into its four component dye colors (corresponding to the termination reactions for each base) and then aligns termination reactions from multiple sequences. Heterozygotes and homozygote mutants can then be identified by the presence of a novel peak in one of the dye colors for a particular sample with a corresponding diminution in height (or absence of) the expected peak in one of the other dye colors for the same sample. *See* Chapter 6 for a detailed protocol for CSA.

### **1.8. Meta-PCR**

The material of choice for analysis in most diagnostic labs is DNA and as many genes are complex in genomic organization—they consist of multiple exons, sequence-based mutation scanning will consist of the analysis of each exon individually unless the gene has a very compact genomic organization. Since the average exon size is only 140 bp (4), each sequencing reaction is capable of sequencing more than an average exon. It is possible to exploit this unused capacity by employing a technique known as Meta-PCR (5). Meta-PCR is a method capable of creating of chimeric DNA molecules. It can amplify and assemble several small exons into a single large DNA fragment, which can then be sequenced in a single sequencing reaction. The conditions for Meta-PCR are not particularly demanding. The only requirement is that the primers used to drive the assembly must be carefully designed and of high purity. However, once synthesized, the primers are used in very small amounts, and last for many assays.

## **2. Materials**

### **2.1. Template Preparation**

1. Ultrafree®-DA columns (Millipore Corporation, Bedford, MA).
2. 50X TAE (low ethylenediaminetetraacetic acid [EDTA]). 2 M Tris-acetate, pH 8.0, 5 mM Na<sub>2</sub>EDTA. Autoclave and store at room temperature. Dilute down to 1X final concentration when using in gels and as electrophoresis buffer.
3. Electro-Fast® Standard 108-lane agarose gel tank (ABgene, Epsom, Surrey, UK).

4. Long Wave transilluminator (365-nm wavelength emission).
5. Agarose.
6. 5X TAE (low EDTA) gel loading buffer. 200 mM Tris-acetate, pH 8.0, 0.5 mM Na<sub>2</sub>EDTA, 50% w/v sucrose, bromophenol blue to color (~5 mg per 100 mL).
7. Centricon®-YM100 columns (Millipore Corporation).
8. MicroClean (Microzone Limited, Lewes, UK).
9. Nucleon QC (Tepnel Life Sciences PLC, Manchester, UK).

## **2.2. Cycle Sequencing**

1. BigDye™ terminator cycle sequencing ready reaction kits (Applied Biosystems [ABI], Foster City, CA).
2. DYEnamic ET terminator cycle sequencing kits (Amersham Pharmacia Biotech, Amersham, UK).
3. BigDye™ 2.5X dilution buffer (200 mM Tris-HCl pH 9.0, 5 mM MgCl<sub>2</sub>). Filter-sterilize and store frozen at -20°C in 1 mL aliquots.
4. DYEnamic ET terminator dilution buffer (cat. no. US84002; Amersham Pharmacia Biotech).

## **2.3. Dye Terminator Removal**

1. Sephadex® G-50 Superfine (cat. no. G-50-50; Sigma-Aldrich Company, Poole, UK).
2. MultiScreen HV plates (cat. no. MAHVN4510; Millipore Corporation).
3. MultiScreen centrifuge alignment frame (cat. no. MACF09604; Millipore Corporation).
4. MultiScreen Column loader (cat. no. MACL09645; Millipore Corporation).
5. 96-well v-bottom plates.
6. AutoSeq G-50 (cat. no. 27-5340-01; Amersham Pharmacia Biotech Ltd.).
7. Hi-Di formamide (cat. no. 4311320; ABI).
8. Formamide-loading solution. De-ionized formamide, Pararosilane dye to color. Store in small aliquots frozen at -20°C.
9. Pararosilane (basic red dye) (cat. no. P-7632; Sigma-Aldrich Company).
10. 95% ethanol.
11. 3 M sodium acetate (pH 5.2).

## **2.4. Long-Read Slab Gel Electrophoresis on the ABI377**

1. 48-cm well-to-read plates.
2. 377 Long-read base caller v3.3.1b2 (available from [www.appliedbiosystems.com/support/software/377/basecallers.cfm](http://www.appliedbiosystems.com/support/software/377/basecallers.cfm)).
3. A modified long-read run module with following parameters:
  - a. Electrophoresis voltage: 3000 V.
  - b. Electrophoresis current: 60.0 mA.
  - c. Electrophoresis power: 40 W.
  - d. CCD offset: 250.

- e. Collection time: 16.0 h.
- f. Gel temperature: 45°C.
- g. Laser power: 40.0 mW.
- h. CCD gain 2.
4. 20X Tris-HCl TAPS EDTA (TTE); 1 M Tris, 1 M TAPS, 20 mM Na<sub>2</sub>EDTA. Check that the pH is within the range 8.0–8.6. Store at room temp for up to 4 wk.
5. Urea.
6. 40% PAGE-PLUS concentrate (cat. no. E-562–100 mL; Amresco Inc, Solon, OH).
7. 10% Ammonium persulfate (AMPS). Prepare fresh daily and store at +4°C.
8. TEMED (N,N,N', N'-tetramethylethylenediamine). Store at room temperature. Discard 6 mo after opening.
9. Amberlite MB50 ion-exchange resin (cat. no. A-5710; Sigma-Aldrich Company).
10. Reusable filter holder with receiver (cat. no. 300-4000; Nalge Nunc International, Rochester, NY).
11. 0.2 μM cellulose nitrate filter (cat. no. DS0200-4020; Nalge Nunc International).

### **2.5. Long-Read Capillary Electrophoresis on the ABI3100**

1. 80-cm capillary array (cat. no. 4319899; ABI).
2. 80-cm capillary software support files (cat. no. 4331844; Applied Biosystems).
3. 3100 POP-4 polymer (cat. no. 4316355; ABI).
4. ABI PRISM<sup>®</sup> 3100 Data collection software version 1.0.1 and Sequencing analysis software version 3.7—generally pre-installed on PC supplied with the instrument (ABI).

### **2.6. Universal Sequencing Primers**

“Universal” primer sequences may be added as 5′ tails to your PCR primers. The following sequences are based on the M13 Universal primer but with a higher GC content, so that primer annealing during sequencing can be carried out at a higher temperature, thus leading to higher specificity.

UNISEQ F: 5′ GTA GCG CGA CGG CCA GT 3′  
UNISEQ R: 5′ CAG GGC GCA GCG ATG AC 3′

### **2.7. Sequence Analysis Programs**

1. Sequencher v3.0 (Genecodes Corporation; information and demo for download available at <http://www.genecodes.com/>).
2. Pregap4 and Gap4. Component programs of the Staden Package (available for download at <http://www.mrc-lmb.cam.ac.uk/pubseq/>).
3. Phred for Windows (CodonCode Corporation; information and demo for download available at <http://www.codoncode.com/>).

### 3. Methods

#### 3.1. Template Preparation

Template preparation is a key stage in the process of fluorescent sequencing. It is important to optimize the template PCR prior to sequencing to ensure that the PCR yields a single DNA fragment at high copy number. Using one of the proprietary “Hot-Start” polymerases (e.g., Platinum Taq, AmpliTaq Gold) for your template PCR amplifications is a good way to improve template DNA quality. There are a multitude of methods available for purifying PCR template prior to sequencing. The following are only a small selection of methods known from direct experience to be effective. A brief summary of each method’s respective pros and cons is also given. The choice of method will depend on throughput, the quality of sequence required, and the laboratory budget.

##### 3.1.1. Ultrafree<sup>®</sup>-DA

This protocol is a rapid and effective method of gel purification. It is based on a gel squeeze. It yields high-quality sequencing-grade DNA and allows the purification of the fragment of interest, excluding most nonspecific amplicons. The cost per sample is reasonable (approx \$1–45). However, the relatively low concentrations of agarose required for this method make it less suitable for purification of fragments less than 200 bp.

1. PCR-amplify the DNA fragment in a volume of 20  $\mu\text{L}$ .
2. Prepare a 1% agarose gel with 1X TAE (low EDTA) as the electrophoresis buffer, and add ethidium bromide to the molten agarose to achieve a final concentration of 0.5  $\mu\text{g}/\text{mL}^{-1}$ .
3. Add 5  $\mu\text{L}$  5X TAE (low EDTA) loading buffer to the PCR-amplified samples.
4. Load the samples onto the gel and electrophorese in 1X TAE (low EDTA) running buffer until it has resolved adequately.
5. Place the gel onto a long-wave transilluminator and excise the bands using a clean scalpel blade in a minimum volume of agarose (*see Note 1*).
6. Place the gel slice into the top of the Ultrafree<sup>®</sup>-DA column and centrifuge at 5000g for 10 min in a benchtop centrifuge.
7. The filter unit can now be removed from the Ultrafree<sup>®</sup>-DA column. The filtrate remaining in the collection vial is ready for sequencing. It should be stored at  $-20^{\circ}\text{C}$  prior to sequencing, and is stable for several months.

##### 3.1.2. Centricon<sup>®</sup>-YM100

This protocol produces high-quality sequencing-grade DNA quickly. DNA is purified by dilution/filtration, and recoveries of fragments greater than 250 bp are good. The drawbacks are that the columns are quite expensive (approx \$3–90 each). They are also quite large, and as a result they cannot be

spun in most benchtop centrifuges. An advantage of this method is that primer dimer is removed efficiently. However, low mol-wt DNA (<150 bp) is not retained well by the filter. As a result, they are unsuitable for purifying low mol-wt fragments.

1. PCR-amplify the DNA fragment in a vol of 40  $\mu$ L.
2. Assemble a Centricon<sup>®</sup>-YM100 column according to the manufacturer's instructions, and fill the sample reservoir with 2 mL of dH<sub>2</sub>O.
3. Transfer the whole PCR reaction into the sample reservoir; avoid carrying over any mineral oil.
4. Centrifuge in a (fixed angle) rotor at 1,000g for 10 min.
5. Invert the column, placing the end with the retentate vial into the filtrate vial.
6. Centrifuge at 1000g for 4 min. Decant the retentate from the retentate vial into a clean, fresh microcentrifuge tube (there should be approx 40  $\mu$ L). The retentate is now ready for sequencing. If not used immediately, the filtrate should be stored at  $-20^{\circ}\text{C}$ .

### 3.1.3. Nucleon QC

This protocol also yields good-quality sequencing-grade DNA. The procedure is quick, and the cost per sample is cheap (approx \$0–95). Drawbacks are that care must be taken in handling the resin, as this can be easily damaged and some loss of DNA on the column is to be expected. Loss is proportionately greater with lower starting DNA concentrations.

1. PCR-amplify the DNA fragment in a vol of 30–40  $\mu$ L.
2. Fill one column per sample with 600  $\mu$ L of re-suspended SX resin (*see Note 2*).
3. Centrifuge the column at 750g for 2 min.
4. Remove the liquid that accumulates in the collection vial.
5. Place the whole PCR reaction carefully onto the outer section of the resin slope.
6. Centrifuge again at 750g for 5 min.
7. Dispose of the filter insert containing the resin. The filtrate is now ready for sequencing. If not used immediately, the filtrate should be stored at  $-20^{\circ}\text{C}$ .

### 3.1.4. microClean

Probably the cheapest small-scale method of purification (cost per sample approx \$0–60). This method works on the principle of selective precipitation. The main disadvantage is that the PCR reactions must be clean, and the precipitant does seem to be sensitive to overall salt concentration.

1. If mineral oil was used to cap the PCR amplification, remove the aqueous phase to a separate microcentrifuge tube.
2. Add an equal volume of microClean solution to the template DNA, and mix briefly but thoroughly by vortexing.

3. Leave the solution to stand at room temperature for 5 min.
4. Spin down the precipitate at maximum speed for 5 min in a benchtop centrifuge (*see Note 3*).
5. Carefully remove the supernatant with a pipet tip.
6. Briefly spin again and remove all traces of liquid.
7. Resuspend the DNA pellet (which may not be visible) by adding an equivalent volume of sterile dH<sub>2</sub>O and vortexing briefly.
8. The solution is now ready for sequencing. If not used immediately, it should be stored at  $-20^{\circ}\text{C}$ .

### 3.1.5. High-Throughput Gel Purification with MultiScreen HV Plates

This method yields sequencing-grade DNA of the highest quality, equivalent to that achieved using Ultrafree<sup>®</sup>-DA columns (*see Subheading 3.1.1.*). The method is based on gel purification followed by elution from the gel by a freeze/squeeze. Since the template is gel-purified, no nonspecific amplicons are carried over into the sequencing reactions, and this produces very low backgrounds. The method is also inexpensive—approx \$0–20 per sample. The drawback of this method is that it is relatively labor-intensive, as the bands must be manually excised from the gel, and there can also be variability in recovery yields.

1. PCR-amplify batches of samples in 96-well microtiter plates in 20- $\mu\text{L}$  volumes.
2. Prepare a 110-mL 1% 1X TAE (low EDTA) agarose gel in an Electro-Fast<sup>®</sup> 108 (ABgene Ltd) lane tank, add ethidium bromide to the molten agarose to achieve a final concentration of 0.5  $\mu\text{g}/\text{mL}^{-1}$ .
3. Once the gel is set, add 1X TAE (low EDTA) running buffer to the tank until the surface of the gel is just submerged.
4. Add 5  $\mu\text{L}$  5X TAE (low EDTA) loading buffer to each PCR-amplified sample.
5. Load and electrophorese the samples at 100 V until the fragments have resolved.
6. Transfer the gel onto a long-wave UV transilluminator and excise the bands using a clean scalpel blade (*see Note 1*).
7. Place each fragment into a separate well of a Multiscreen HV plate.
8. Place the Multiscreen HV plate into a freezer at  $-20^{\circ}\text{C}$  or below until the gel slices have completely frozen (usually at least 1 h).
9. Place a 96-well low-profile “catch plate” within a 96-well tray for support. Place a centrifuge alignment frame upon the “catch plate.” Finally, place the Multiscreen HV plate on top of the alignment frame.
10. Using a weighed balancing “catch plate” assembly, centrifuge the Multiscreen HV plate in a swinging bucket centrifuge at 3000g for 10 min.
11. The filtrate is now ready for sequencing. If not used immediately, the filtrate should be stored at  $-20^{\circ}\text{C}$ .

## 3.2. Dye Terminator Cycle Sequencing

### 3.2.1. BigDye™ Terminator Cycle Sequencing Kits

BigDye™ fluorescent labels are based on cooperative donor and acceptor dyes. In the v2.0 kits, a universal fluorescein donor dye is covalently attached to one of four dichlororhodamine acceptor dyes, depending on which dideoxynucleoside residue is labeled. The argon laser within the sequencer emits ultraviolet (UV) light at 488 nm, the absorbance maximum of the fluorescein donor dye. The donor dye absorbs the laser UV light and enters an excited state. When the donor dye de-excites, it passes its energy with high efficiency onto the acceptor dye, which in turn becomes excited and then fluoresces with its characteristic emission spectra. This arrangement leads to a two- to three-fold greater increase in fluorescence than that achieved by the dichlororhodamine dyes alone, which have an absorbance maxima that do not precisely match that of the laser. This principle is also used by the “DYEnamic ET” sequencing kits marketed by Amersham Pharmacia. At the present time, the v2.0 kits are in the process of being withdrawn from sale and replaced by the v3.0 kit, which operates on the same principle but uses slightly different dyes. The sequencing enzyme used in the BigDye™ kits is an engineered form of *Taq* polymerase known as AmpliTaqFS, which has an amino acid substitution in the active site. This causes the enzyme to incorporate the dye-labeled dideoxynucleosides more evenly than native *Taq* polymerase.

BigDye™ sequencing kits come as a single tube pre-mix; the user must add template and primer. The standard volume of a BigDye™ sequencing reaction is 20  $\mu\text{L}$ . However, this laboratory and many other users save on consumable costs by either reducing the volume of the sequencing reaction or diluting down the sequencing reagent using a sequencing diluent. Reducing the volume to 10  $\mu\text{L}$  on all but the most demanding templates still provides sufficient sequencing reaction to run at least twice on a 377 sequencer, and many more times on a capillary sequencer e.g., 310, 3100, or 3700. The sequencing diluent can also reduce the consumption of sequencing kits up to fivefold (*see Note 4*).

1. For best results, quantitate the template DNA before sequencing. For most PCR products between 200 and 1000 bp, 20–100 ng of DNA gives good signal intensities (*see Note 5*).
2. Make a dilution of the sequencing primer to 3.2  $\mu\text{M}$  in sterile  $\text{dH}_2\text{O}$ .
3. Individual sequencing reactions may then be prepared as follows in 200  $\mu\text{L}$  microcentrifuge tubes, 24- or 96-well plates:
  - a. 1  $\mu\text{L}$  Sequencing primer at 3.2  $\mu\text{M}$ .
  - b. 4  $\mu\text{L}$  BigDye™ Ready Reaction premix.

- c. 20–100 ng of template DNA.
- d. dH<sub>2</sub>O to 10  $\mu$ L
4. When using the BigDye™ dilution buffer, this should be substituted directly for any omitted BigDye™ premix. The volume of BigDye™ premix plus dilution buffer should always be 40% of the total reaction volume. For example, with 2  $\mu$ L of BigDye™ premix per 10  $\mu$ L sequencing reaction 2  $\mu$ L of the 2.5X dilution buffer should be added. With 3  $\mu$ L of BigDye™ premix 1  $\mu$ L of 2.5X dilution buffer should be added per 10  $\mu$ L reaction.
5. For best results, make a master mix of BigDye™ Ready Reaction premix, sequencing primer, and dH<sub>2</sub>O. This can then be added to each template DNA. A master mix minimizes pipetting inaccuracies and reduces variability between samples.
6. Mix samples by vortexing, and spin down by centrifugation.
7. Place reactions on a thermal cycler and cycle under the following conditions:
  - a. 94°C: 1 min: Initial denaturation.  
30 cycles of:
  - b. 96°C: 10 s
  - c. 50°C: 20 s (*see Note 6*).
  - d. 60°C: 4 min.
  - e. Then hold at 4°C.
8. The sequencing reactions should be stored at –20°C if they are not being processed immediately.

### 3.2.2. DYEnamic ET Cycle Sequencing Kits

Although the principle of cooperative absorbance and fluorescence of the dye labels is identical to that used in the BigDye™ kits the sequencing enzyme, Thermo-Sequenase II, has been engineered in a different way and the dyes have slightly different emission and mobility properties. This necessitates the creation of a specific matrix (ABI 377) or spectral file (ABI 3100) and the installation of a specific mobility correction file for these kits. As is the case with the BigDye™ kits, a dilution buffer can be used to economize on cost. DYEnamic ET dilution buffer, however, can only be purchased from the manufacturer (*see Subheading 2.2.* for details, also *see Note 4*). Many of the details of the protocol mirror those of the BigDye™ protocol in **Subheading 3.2.1**. Nevertheless, there are some critical differences. For ease of use, the protocol is given in full.

1. For best results, quantitate the template DNA before sequencing. For most PCR products between 200 and 1000 bp, 20–100 ng of DNA gives good signal intensities (*see Note 5*).
2. Make a dilution of the sequencing primer to 2.5  $\mu$ M in sterile dH<sub>2</sub>O.
3. Individual sequencing reactions may be made up as follows in 200  $\mu$ L microcentrifuge tubes, 24- or 96-well plates:
  - a. 1  $\mu$ L Primer at 2.5  $\mu$ M
  - b. 4  $\mu$ L Sequencing reagent premix.



- c. 20–100 ng of template DNA.
- d. dH<sub>2</sub>O to 10  $\mu$ L.
4. For best results, make a master mix of Ready Reaction premix and sequencing primer and dH<sub>2</sub>O. This can then be added to each template DNA. A master mix minimizes pipetting inaccuracies and reduces variability between samples.
5. Mix samples by vortexing, and spin down by centrifugation.
6. Place reactions on a thermal cycler and cycle under the following conditions:
  - 30 cycles of:
    - a. 95°C: 20 s.
    - b. 50°C: 20 s (*see Note 6*).
    - c. 72°C: 1 min.
  - Then hold at 4°C.
7. The sequencing reactions should be stored at –20°C if they are not being processed immediately.

### 3.3. Dye Terminator Removal

Several methods are available for removal of dye terminators. There are differences between sequencing chemistries, so methods are not necessarily interchangeable. The cheapest methods involve precipitation of the terminators. Precipitation can be quite effective; however, it can lead to variability both between samples and batches, as the efficiency of removal is critically dependant on both the ethanol concentration, the time period of precipitation as well as the centrifugation conditions.

#### 3.3.1. Sodium Acetate/Ethanol Precipitation of BigDye™ Terminators

This protocol can be used for precipitation in both 96-well trays and individual microcentrifuge tubes. This procedure is quite tolerant of variability. However, it is unsuitable for capillary electrophoresis instruments because the sodium acetate interferes with the sample loading process.

1. To a 10- $\mu$ L sequencing reaction add 1  $\mu$ L of 3 M sodium acetate (pH 5.2) and 25  $\mu$ L of 95% ethanol.
2. Thoroughly mix the reaction and leave to stand at room temperature for 15 min.
3. Centrifuge the microtiter plate or microcentrifuge tubes at 3000g for 30 min (*see Note 3*).
4. Carefully remove all the supernatant. For microcentrifuge tubes, use a pipet tip. For 96-well trays invert over a folded paper towel and spin inverted at 300g for 1 min.
5. Add 100  $\mu$ L of 70% ethanol to each pellet. Do not resuspend the pellet.
6. Re-centrifuge the microcentrifuge tubes or trays at 3000g for 5 min.
7. Repeat **step 4**. Allow the pellet to dry by leaving the tubes/plate open to the air covered by a disposable tissue for 10 min.
8. Resuspend the pellet in 1  $\mu$ L of formamide loading solution.
9. If samples are not loaded immediately, they should be stored at –20°C.

### 3.3.2. Ethanol-Precipitation of BigDye™ Terminators

This protocol is an adaptation of the precipitation protocol in **Subheading 3.3.1**. The absence of sodium acetate makes it suitable for use with capillary sequencers. However, it is less tolerant of variability than the first protocol, and some carryover of terminators is to be expected.

1. To a 10- $\mu$ L sequencing reaction add 8  $\mu$ L of dH<sub>2</sub>O and 32  $\mu$ L of 95% ethanol.
2. Thoroughly mix and leave to stand at room temperature for 15 min.
3. Centrifuge the microtiter plate or microcentrifuge tubes at 3000g for 30 min (*see Note 3*).
4. Carefully remove all the supernatant. For microcentrifuge tubes, use a pipet tip. For 96-well trays, invert over a folded paper towel and spin inverted at 300g for 1 min.
5. Add 100  $\mu$ L of 70% ethanol to each pellet. Do not resuspend the pellet.
6. Re-centrifuge the microcentrifuge tubes or trays at 3000g for 5 min.
7. Repeat **step 4**. Allow the pellet to dry by leaving the tubes/plate open to the air covered by a disposable tissue for 10 min.
8. Re-suspend the pellet in 20  $\mu$ L of Hi-Di formamide-loading solution or alternatively in 1  $\mu$ L of formamide if running on a slab gel sequencer.
9. If samples are not loaded immediately, they should be stored at  $-20^{\circ}\text{C}$ .

### 3.3.3. Sodium Acetate/EDTA/Ethanol Precipitation of DYEnamic ET Terminators

This protocol is suitable for terminator removal for both slab gel and capillary sequence analysis. However, it has only been tested with DYEnamic ET terminators.

1. To a 10- $\mu$ L sequencing reaction, add 1  $\mu$ L of 1.5 M sodium acetate (pH 8.0)/250 mM EDTA solution and 40  $\mu$ L of 95% ethanol.
2. Thoroughly mix the reaction and leave to stand at room temperature for 15 min.
3. Centrifuge the microtiter plate or microcentrifuge tubes at 3000g for 30 min (*see Note 3*).
4. Carefully remove all supernatant. For microcentrifuge tubes, use a pipet tip. For 96-well trays, invert over a folded paper towel and spin inverted at 300g for 1 min.
5. Add 100  $\mu$ L of 70% ethanol to each pellet. Do not resuspend the pellet.
6. Re-centrifuge the trays or microcentrifuge tubes at 3000g for 5 min.
7. Repeat **step 4**. Allow the pellet to dry by leaving the tubes/plate open to the air covered by a disposable tissue for 10 min.
8. Resuspend the pellet in 1  $\mu$ L of formamide-loading solution if loading on a slab gel sequencer, or alternatively in 20  $\mu$ L of Hi-Di formamide if running on a capillary sequencer.
9. If samples are not loaded immediately, they should be stored at  $-20^{\circ}\text{C}$ .

### 3.3.4. AutoSeq50 Columns

These pre-prepared sephadex G-50 columns are useful for purifying small numbers of samples free from dye terminators. The quality of sequencing data achieved is comparable to that achieved in **Subheading 3.3.5.** with MultiScreen HV plates.

1. Resuspend the resin in the column by gently vortexing.
2. Open the cap about one-quarter of a turn and break off the tab at the base by twisting.
3. Place the column into a suitable 1.5-mL microcentrifuge tube and spin for 1 min at 2000g in a microcentrifuge.
4. Place the column in a fresh 1.5-mL microcentrifuge tube and carefully add the sequencing reaction to the center of the resin column.
5. Centrifuge again for 1 min at 2000g. The purified sample is collected in the microcentrifuge tube.
6. Evaporate the purified sample to dryness in a vacuum dryer.
7. Resuspend the pellets in either 20  $\mu\text{L}$  of Hi-Di formamide (for capillary sequencers) or 1  $\mu\text{L}$  of formamide loading solution (for slab gel sequencers).
8. If samples are not loaded immediately they should be stored at  $-20^{\circ}\text{C}$ .

### 3.3.5. G-50 Filled MultiScreen 96-Well HV Plates

This protocol is a high-throughput method which is low-cost and also yields sequence data virtually uncontaminated by dye blobs, thus permitting reads from the end of the primer. This is our laboratory's preferred method for high-throughput applications.

1. Pour Sephadex G-50 ultra-fine onto the column loader. Using a clean spatula, ensure that all 96 wells are completely filled.
2. Using the scraper, carefully remove any excess G-50.
3. Place a MultiScreen HV plate on top of the column loader, ensuring that the plate and mold are correctly aligned.
4. Invert the MultiScreen HV plate/column loader assembly without allowing them to separate, and place on a benchtop.
5. Firmly tap the column loader to dislodge the Sephadex. Then carefully remove the column loader. All 96 wells of the MultiScreen HV plate should now be filled with the correct amount of G-50.
6. Using a multi-channel pipet, add 300  $\mu\text{L}$  of Milli-Q water to each well of the HV plate. Try to prevent air bubbles being trapped in the G-50. Leave the G-50 to swell for 3 h (*see Note 7*).
7. Once the columns have swollen, take a 96-well v-bottom microplate mounted in a support tray (the catch plate), place a centrifuge alignment frame on top of the catch plate. Finally, place the MultiScreen HV plate on top.

8. Centrifuge the assembly in a microplate rotor at 910g for 5 min.
9. If the sequencing reactions are to be loaded onto a capillary sequencer, the 96-well plate should be emptied of the flowthrough and a further 150  $\mu$ L of Milli-Q water added to each column using a multichannel pipet. The entire assembly should then be centrifuged again at 910g for 5 min.
10. Exchange the 96-well v-bottom microplate in the catch plate for a fresh 96-well v-bottom microplate (the old microplate can be emptied and reused). Next, carefully aliquot each sequencing reaction onto the center of a separate G50 column in the HV plate. Then centrifuge the assembly at 910g for a further 5 min.
11. The sequencing reactions are now suspended in water in the wells of the 96-well v-bottom microplate. Evaporate the water in a vacuum dryer capable of accommodating 96-well plates.
12. Re-suspend the pellets in either 20  $\mu$ L of Hi-Di formamide (for capillary sequencers) or 1  $\mu$ L of formamide loading solution (for slab gel sequencers).
13. If samples are not loaded immediately, they should be stored at  $-20^{\circ}\text{C}$ .

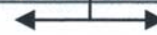
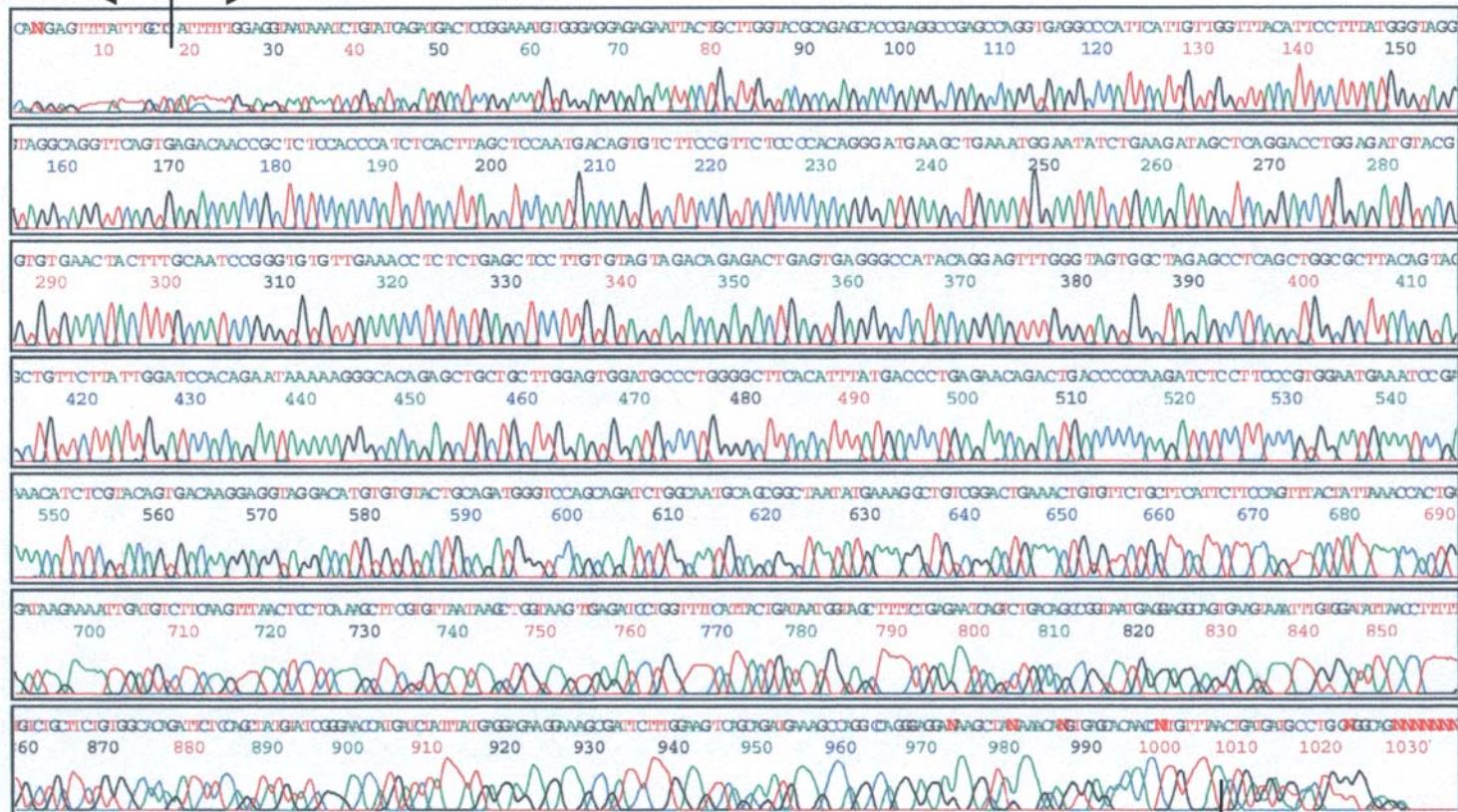
### 3.4. Long-Read Slab Gel Electrophoresis

Significantly longer reads can be achieved on the ABI377 platform with a few modifications to the manufacturer's standard protocols. In our experience, good reads exceeding 900 bp are typical (**Fig. 1**). The use of this protocol can increase efficiency and reduce costs by allowing large exons to be screened in smaller numbers of sequencing reactions, or alternatively, coupled with the use of Meta-PCR to allow the sequencing of multiple small exons within a single larger fragment. Applied Biosystems (ABI) has issued detailed instructions for this protocol in the form of a user bulletin (available at <http://docs.appliedbiosystems.com/pebiiodocs/04315153.pdf>). Assuming that the software components and modifications specified in **Subheading 2.4.** are installed and have been carried out, the protocol is as follows.

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Fig. 1. (*see facing page*) Long-read sequencing data. Sequence of a 1063-bp Meta-PCR fragment from the NF2 gene comprising exons 6,7,8,9 and 10. The template was purified by gel purification using MultiScreen HV plates (*see Subheading 3.1.5.*). The fragment was sequenced using a Universal primer (*see Subheading 2.5.*) and BigDye<sup>TM</sup> terminator v3.0 sequencing chemistry. Unincorporated dye-labeled terminators were removed using G-50 filled MultiScreen 96-well HV plates (*see Subheading 3.3.5.*), and the sequencing reactions were run out using the long-read sequencing protocol on an ABI377 (*see Subheading 3.4.*). The first "N" base call, after an initial mobility correction error early in the sequence, is at nt974. The boundaries between the sequence derived from the primers and the intervening amplified region are marked. The overall base-calling accuracy of the 998bp fragment between the primers is 99.6%.

Primer      Intron 6



Intron 10      Primer



### 3.4.1. Gel Pouring

1. Clean and assemble a set of 48-cm well-to-read plates and 0.2-mm spacers.
2. In a clean glass beaker, combine the following components:
  - a. 18 g urea.
  - b. 5.3 mL 40% PAGE-PLUS concentrate.
  - c. 24 mL dH<sub>2</sub>O.
  - d. 250  $\mu$ L 10% ammonium persulfate (AMPS).
3. Using a magnetic stirrer bar, stir until all the urea crystals have completely dissolved.
4. Add 0.25 g Amberlite MB50 ion-exchange resin, and continue stirring for 5 min.
5. Assemble a Nalgene filter unit with a 0.2- $\mu$ M single-use cellulose nitrate filter.
6. Add 3 mL of 20X TTE to the upper chamber of the filter unit and filter under vacuum.
7. Next, pour the dissolved urea/acrylamide solution into the upper chamber of the filter unit, and draw the solution through the filter under vacuum.
8. Seal the lid of the filter unit and while maintaining the vacuum, allow the solution to thoroughly degas for at least 5 min.
9. Decant the gel solution into a clean 50-mL measuring cylinder.
10. Make the solution up to 50 mL by adding dH<sub>2</sub>O.
11. Pour into a clean glass beaker, add 25  $\mu$ L of TEMED, and swirl to mix. Without delay, pour the gel using a clean 50-mL syringe.
12. Insert a straight edge casting comb and allow the gel to polymerize for at least 1 h before use.
13. Insert an appropriate sharktooth comb. Then place the plates in the gel cassette and the gel cassette in the electrophoresis chamber of the ABI377. Finally, carry out a “plate check.”

### 3.4.2. Gel Electrophoresis

In order to achieve optimum read lengths, samples should be loaded and run into the gel in water rather than electrophoresis buffer (a “water run in protocol”). The following modifications are necessary to the standard gel electrophoresis protocol recommended by ABI.

1. Fill the lower buffer chamber with 600 mL of 1X TTE buffer.
2. Fill the upper buffer chamber with 594 mL of dH<sub>2</sub>O.
3. Select the modified long-read run module on the 377 data collection software.
4. Commence the run and pause *immediately* when electrophoresis commences, then wait approx 10 min for the gel to reach 45°C.
5. Denature the samples by placing on a heated block at 90°C for 3 min, then snap-chill on a bed of crushed ice.
6. Rinse the wells thoroughly with a syringe and needle to remove urea and unpolymerized acrylamide. Stagger-load 0.4  $\mu$ L of the heat-denatured sequencing reaction in the “odd-numbered” lanes (e.g., lanes 1,3,5,7,9 . . . etc.). Commence electrophoresis for 1 min, then pause the instrument (*see Note 8*).

7. Stagger-load the remaining “even numbered” lanes (e.g., lanes 2,4,6,8 . . . etc.). Resume electrophoresis for 1 min, then pause the instrument again.
8. Add 66 mL of 20X TTE to the water in the upper buffer chamber (to make a final concentration of 2X TTE). Thoroughly mix with a 50-mL syringe, taking care to rinse out the wells to remove any residual water in contact with the gel surface.
9. Resume electrophoresis for the remainder of the 16-h run.
10. Once the run is completed, the samples can be tracked and extracted as usual. However, the LR377 basecaller should be selected in the sample manager of the 377 sequence analysis software.

### 3.5. Long-Read Capillary Electrophoresis on the ABI 3100

Read lengths up to 1000 bp can be achieved on the ABI 3100 platform using 80-cm capillaries. As is the case with the ABI 377 long-read protocol, costs can be reduced by allowing large exons to be screened in smaller numbers of sequencing reactions, or alternatively, coupled with the use of Meta-PCR to allow the sequencing of multiple small exons within a single larger fragment. ABI have issued detailed instructions for the installation of the necessary support software in the form of a user bulletin (available at <http://docs.applied-biosystems.com/pebi docs/04331148.pdf>). Instructions for the preparation and running of analytical samples are also provided in this user bulletin. A brief summary protocol is given here:

1. Ensure that the instrument is prepared for the run:
  - that there is sufficient POP-4 polymer loaded in the array-fill and reservoir syringes to complete the run.
  - that the electrophoresis buffer in the cathode and anode buffer chambers is not more than 24 h old.
  - that the water reservoirs are filled with clean dH<sub>2</sub>O.
  - that the polymer block has been cleaned within the last 7 d.
2. Enter details of the samples in a new Plate Record.
3. Select the relevant dye set (dye set “E” for BigDye™ v1 or v2 terminators) in the plate record.
4. Select the appropriate mobility file for the sequencing chemistry you are using in the plate record (“DT3100POP4LR{BD}v1.mob” for BigDye™ terminators).
5. Select the long-read run module “LongSeq80\_POP4DefaultModule” in the plate record.
6. Select the long-read analysis module in the plate record “BC-3100POP4\_80cm\_SeqOffFtOff.saz.” Click “Finish” to save the plate record.
7. If not already done, resuspend the “cleaned up” sequencing reactions in 20 µL of Hi-Di formamide (see Subheadings 3.3.1–3.3.5).
8. Denature the samples by placing on a heated block at 90°C for 3 min and then snap-chill on a bed of crushed ice.
9. Place the plate assembly on the autosampler.

10. Link the relevant pending plate record(s) to the plate(s) loaded on the autosampler.
11. Click the “**Run**” button. The run time is approx 3 h 45 min per set of 16 samples.

### **3.6. Sequence Analysis with the Staden Package**

Anyone who has visually analyzed sequence electropherograms from potentially heterozygous samples soon realizes that they have a problem of too much data. The sheer volume of information in the form of each individual peak, its height, the called base, and the relative heights of the uncalled peaks must be considered when attempting to identify mutations. It soon becomes apparent that automated or assisted analysis is necessary for anything other than the lowest-throughput applications. Several software packages can be used for heterozygote analysis. This laboratory’s favored solution utilizes trace subtraction, which can be carried out using the Gap4 database viewer, available as part of the Staden package of programs (Bonfield et al., 1998). At the present time, the latest full version of the Staden package (v2000.0) is available for use with both a Windows (Win9x/NT/2000) and Unix/Linux operating system. Please note that the guidance notes that follow have only been tested on the Windows version.

#### **3.6.1. Transfer of Analyzed Sequence Files into the Staden Package**

1. If using collection software that is running on a Windows NT platform, analyzed ABI sequence files can be imported directly into the Staden package.
2. If using collection software running on a Macintosh platform, the analyzed ABI sequence files must be converted into PC format prior to the import into Staden. This can be achieved in two ways—either using the Mac to PC converter supplied with Sequencing analysis v3.5-3.7, or alternatively, manually.
3. If a Mac to PC converter is already installed on the Macintosh computer, simply drag the folder containing the files to be converted onto the desktop icon for the converter program. The program automatically assigns the files a PC-compatible identity that includes the removal of spaces from the filenames and the addition of a “.ab1” suffix. The files may now be transported to the PC running Staden using a PC formatted disc, via a local network or E-mail.
4. Alternatively, to manually convert the sequencing electropherograms, first transfer the files either onto a PC-compatible disc (e.g., zip disk, CD-RW) or onto the PC running the Staden package, using a local network or E-mail. Remove any spaces and dots from the file names by deleting or replacing with a character accepted in filenames by Windows operating systems, such as underscore. Finally add the suffix “.ab1” to all the sequence files.
5. Once the files have been converted, they are ready for import and analysis by the Staden package. However, it is **essential** that all folder names containing the files (including master folders further up the hierarchy) **do not** contain any



spaces—e.g., “**Staden sequences**” as a folder name is not acceptable but “**Staden\_sequences**” with an underscore replacing the space is acceptable.

### 3.6.2. Assembly and Alignment of Sequences into Contigs Using Pregap4

The first stage of analysis is the import and alignment of raw sequencing files. Pregap4 is a program that prepares raw sequencing files and then aligns them into contigs that are viewed using a second program Gap4. For the purposes of diagnostic sequencing, a contig of sequences to be assembled by Pregap4 will typically be a single-amplimer-sequenced, using a single primer from a set of test samples along with a reference normal control. The corresponding reverse or complementary sequences are typically batched separately and assembled into a separate contig. There are many modules available within Pregap4 that carry out other useful processes on the sequencing data, e.g., quality clipping, or vector sequence removal. It is not possible within the remit of the current article to cover all these functions. Readers who wish to know more about the full capabilities of the Staden package are advised to consult the Staden package home page at <http://www.mrc-lmb.cam.ac.uk/pubseq/>, where full instructions and support are available.

1. First run Pregap4 by clicking on the appropriate desktop icon or from the Start menu on the Windows taskbar.
2. Click on the “**Add files**” button and select the files for analysis (**Fig. 2A**).
3. Click on the “**Configure Modules**” tab. A list of modules is displayed. Those that are active have a corresponding tick, and disabled modules have a cross. If not already enabled, select the modules “**ALF/ABI to SCF conversion**” by using the cursor and “**Initialize Experiment Files**,” and change their status so that a tick is displayed to the left of the corresponding text (**Fig. 2B**).
4. If the basecaller Phred is installed on the workstation or network, this module may also be selected as active. Phred is considered to be a superior basecaller to those supplied with commercial sequencing software. Base-calling with Phred also allows an individual confidence score to be calculated for each base, providing an estimate of the sequence quality (6,7).
5. Select “**Gap4 shotgun assembly**.” Once Gap4 shotgun assembly is selected, parameters for assembly of the contig must be set. We recommend that the parameters for comparison of long-read sequencing data are set as follows:
  - “**Minimum exact match = 15**”
  - “**Maximum number of pads = 75**”
  - “**Maximum percentage mismatch = 25.0**”A name must also be given for the contig database “Test” was used in the example (**Fig. 2B**). The “**Create new database**” box must also be checked.
6. Ensure that all other modules are disabled—e.g., that they all have a cross displayed to their left-hand side.

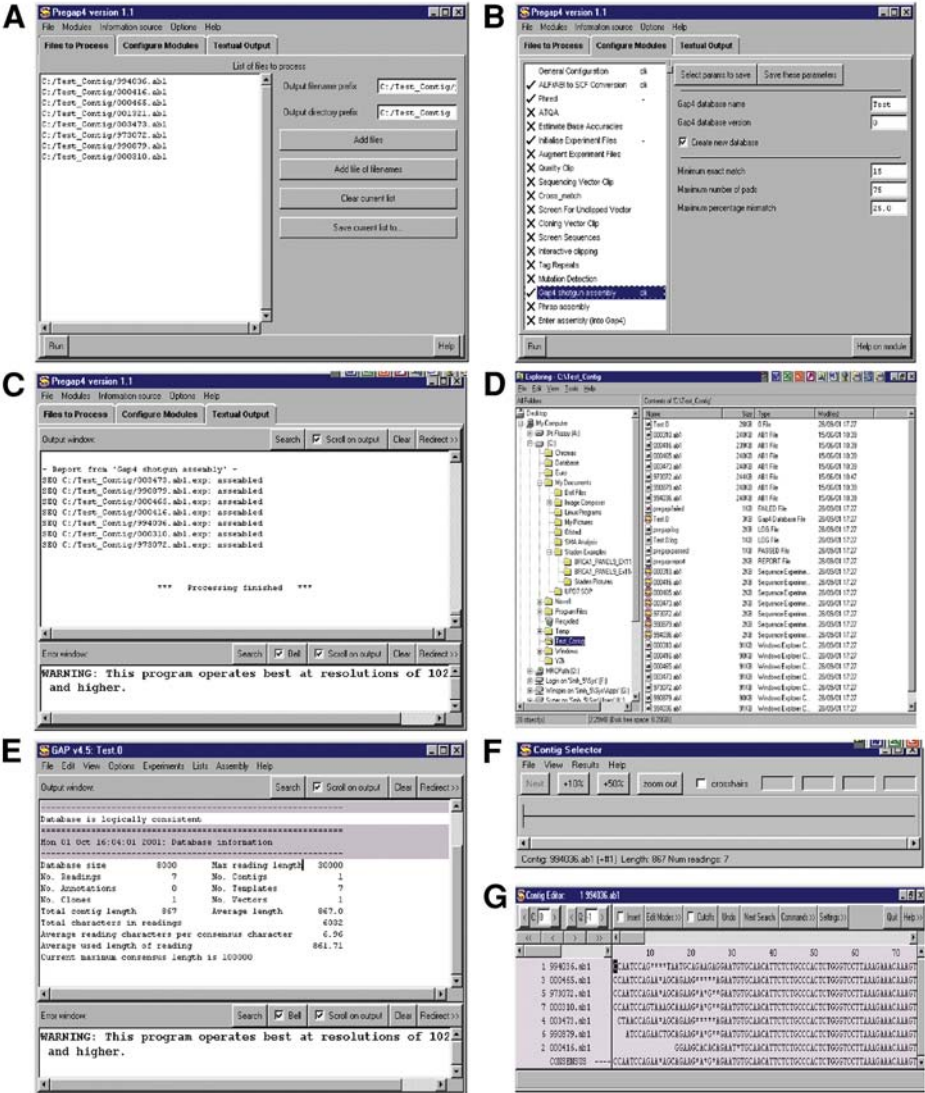


Fig. 2. Operation of Pregap4 and Gap4. (A) Import of files into Pregap4; appearance of window after addition of files for processing. (B) The Pregap4 module editor; recommended settings for alignment of re-sequencing data for point mutation analysis. (C) Appearance of Pregap4 after successful processing of files. (D) Files created by Pregap4 as visualized using “Windows Explorer.” (E) Appearance of the Gap4 opening window. (F) The Contig Selector, which appears alongside the Gap4 opening window when a Gap4 database file is opened. (G) The Contig Editor window with the sequences of the constituent files.

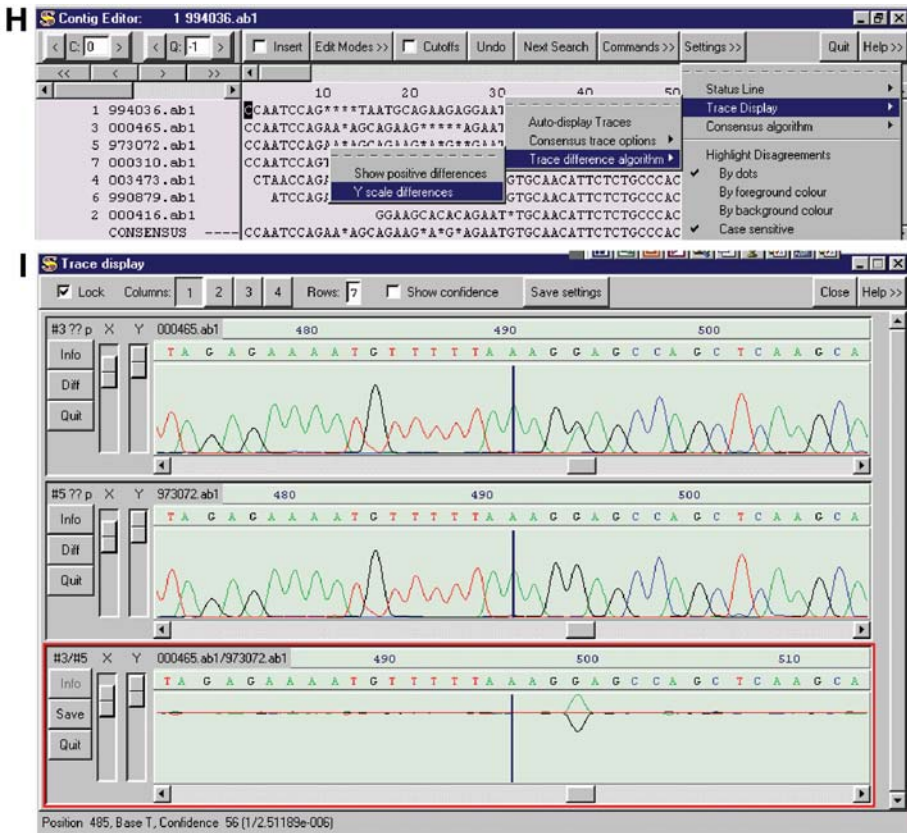


Fig. 2. (continued) (H) Selection of the trace difference algorithm “Y scale differences.” (I) Trace display. Display of two sequence traces and a difference trace formed from subtraction between them. The upper trace is a heterozygote, and the position of the heterozygous base is clearly visible in the difference trace.

7. Click the “Run” button. A series of textual messages should scroll through, and after a few seconds a message should be displayed and the last line should state “processing finished” (Fig. 2C).
8. If the folder containing the original sequencing files is now inspected using the program “Windows Explorer,” a whole new set of files should now be present (Fig. 2D). For each ABI sequence file processed, there should be a corresponding Sequence Experiment File and an SCF file. Among the other files created by Pregap4, there should also be a Gap4 database file with the name you specified in Pregap4. This file may now be examined using Gap4 to identify the presence of mutations.

### 3.6.3. Examination of Aligned Sequence Data for Mutations Within Gap4

Gap4 is a complex and powerful genome assembly program. The functions that are exploited to identify mutations in this chapter use only a very small proportion of the total available. Once again, as with Pregap4, readers who are interested to know more about the full capabilities of the Gap4 are advised to consult the Staden package home page at <http://www.mrc-lmb.cam.ac.uk/pubseq/>, where full instructions and support are available.

1. Run Gap 4 by clicking on the appropriate desktop icon or from the Start menu on the Windows taskbar (*see Note 9*).
2. Select “*Open*” from the “*File*” menu, and then select the newly created Gap4 database file from your sequencing folder. A screen that looks like **Fig. 2E** should become visible.
3. The contig selector should also be visible as a separate window (**Fig. 2F**). Each contig should be visible as a line delimited by a perpendicular bar. A single set of sequencing data from the same fragment and using the same primer should yield a single contig. Occasionally, a frameshift mutation within a sample can cause it to be placed in a separate contig, which can be resolved by inspection of the relevant sequence within Gap4. The number of sequences incorporated within each contig and the length of contig are also displayed within the contig selector window when the mouse pointer is placed over the contig selector.
4. Edit the contig by right mouse clicking the contig bar and selecting “*Edit contig*” from the pop-up menu.
5. The Contig Editor window should now appear (**Fig. 2G**). Next, the Trace Display settings must be altered to optimize trace subtraction. To do this, click the “*Settings*” button and select “*Trace display*” > “*Trace difference algorithm*” > “*Y scale differences*” from the series of menus (**Fig. 2H**) (*see Note 10*).
6. Double clicking a base from one of the sequences present in the Contig Editor displays the corresponding sequence trace. Double clicking another sequence allows both sequences to be displayed, aligned above each other and locked together (*see Notes 11 and 12*).
7. A difference trace composed solely of the differences between two sequences can now be created by first clicking the “*Diff*” button on the left side of one of the sequence traces. Move the cursor onto the second sequence, and a cross icon should appear. Clicking with the cross on the window for a second sequence causes a third window to open, which contains the difference trace (**Fig. 2I**) (*see Note 13*).
8. The difference trace and the originating sequences can now be inspected together by scrolling along, using either the navigation arrows or the locator bar situated beneath any of the sequence windows. Mutations can clearly be seen as deflections within the difference trace above and below the central line (**Fig. 2I**) (*see Notes 14 and 15*).

### 3.7. Sequence Analysis with Sequencher

Like Pregap4 and Gap4 in the Staden package, Sequencher is a program that is capable of importing, manipulating, and aligning raw automated sequencing data into contigs. Unlike Pregap4 and Gap4, all functions are carried out by the same program. Sequencher is available in both Macintosh and Windows versions. Although the following instructions are based on the Macintosh version of Sequencher, the differences between the two versions are minimal.

1. First run Sequencher by clicking on the appropriate desktop icon or from the Apple menu items. The Sequencher project window should be visible (**Fig 3A**).
2. Next select **"File" > "Import&Export" > "Import Sequences"** from the pull down menu. Locate and select the files or folder of interest and click **"Open"** and click **"Done"** when complete. The selected files should now appear in the project window (**Fig. 3B**).
3. Next check the **"Assembly Parameters"** button and alter the conditions to your personal preferences. Suggested values are as follows:
  - **"Dirty Data algorithm"**
  - **"Min. match = 75%"**
  - **"Min. overlap = 20"**
  - **"Max Loop Size = 2"**
4. Select the sequence files to be assembled from the project window using the mouse and click the **"Assemble Automatically"** button.
5. The sequences should now be aligned and represented by a single contig icon in the project window (**Fig. 3C**).
6. Double click on the contig icon, the **"Contig Overview"** window should become visible (**Fig. 3D**). Click the **"Bases"** button, and the sequence data should appear in the window along with the corresponding file names (**Fig. 3E**).
7. Next, choose **"Sequence" > "Call Secondary Peaks"** from the pull-down menu. A dialog box appears that allows a threshold to be set on how significant a secondary peak should be to be flagged up. The actual level chosen will depend on the quality of your sequence data, but in our experience the level should be at a maximum of 30%. If set any higher, there is a significant possibility of missing some heterozygote substitutions (*see Note 16*). Select the **"Search All Bases"** button and click OK.
8. On the Contig Overview window, click anywhere on the consensus sequence, a bar should highlight over all the sequences in the contig. Now click the **"Show Chromatograms"** button. A new window should open containing all the chromatograms tiled and aligned above each other (**Fig. 3F**). Now click at the beginning of the consensus base sequence and depress the Apple and E keys simultaneously. The display should now toggle to the next base in the contig with a significant secondary peak (**Fig. 3F**). Each anomaly can be visually inspected





to determine whether it is a genuine heterozygote. The Apple and E keys can now continue to be depressed until all the secondary peaks have been inspected and the end of the sequence has been reached.

### 3.8. Troubleshooting Guide

1. A poor-quality, impure sequencing primer gives rise to a characteristic sequencing electropherogram. Since the usual impurity is an N-1 failure product, a peak is most often preceded by a subsidiary minor peak (**Fig. 4**). Re-synthesis of the primer is the only solution. HPLC purification of sequencing primers, although expensive, should eliminate this problem. A less expensive alternative is to use a manufacturer of good-quality oligonucleotides.
2. A common problem observed with sequencing data is an electropherogram that commences strongly but then declines in intensity until there is no signal. This is most clearly observed on the raw data image (**Fig. 5**). It is usually a result of carry-over of salt from the template preparation stage. To minimize the occurrence of this problem, use a template preparation method that avoids addition of Na<sup>+</sup> ions. All the methods given in **Subheading 3.1.** avoid the addition of salt, and are thus not prone to this problem.
3. A noisy electropherogram for the initial 20–30 nt followed by clear data is usually caused by carryover of primer-dimer from the template preparation PCR (**Fig. 6**). If clear resolution for the initial sequence is required, then use a template purification method that involves gel purification (*see Subheading 3.1.1.* or **3.1.5.**). Alternatively, the method based on Centricon<sup>®</sup>-YM100 columns (*see Subheading 3.1.2.*) minimizes primer dimer carryover since the mol-wt cut-off of the columns is of the order of 100 bp.
4. Occasionally, a long mononucleotide repeat is encountered that proves difficult to sequence across because of template slippage (**Fig. 7**). When this occurs, it is usually possible to work out what the base following the repetitive tract is, even if the sequence is not published. Once this has been determined, the fragment can simply be re-sequenced with a primer comprising the repeated nucleotide anchored at its 3' end, with the nucleotide at the end of the repeated element.

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Fig. 3. (*see opposite page*) Operation of Sequencher. **(A)** The Sequencher project window visible when the program is initially run. **(B)** Appearance of the project window, with files imported ready for contig assembly. **(C)** Once sequences have been assembled, a contig is now represented in the project window. **(D)** The contig overview window, visible when a contig icon is double clicked in the project window. **(E)** Sequence data, which becomes visible upon clicking the “Bases” button in the contig overview window. **(F)** Electropherograms displayed from a contig of four sequences. Two of the sequences have a heterozygote base (the second and fourth), which has been highlighted after secondary peaks have been called. The fourth sequence does not quite fit within the program window.

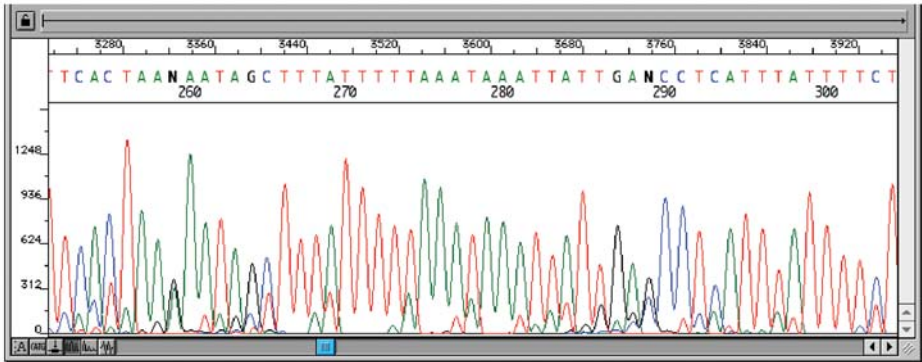


Fig. 4. A sequence electropherogram showing subsidiary peaks arising from N-I contamination of the primer.

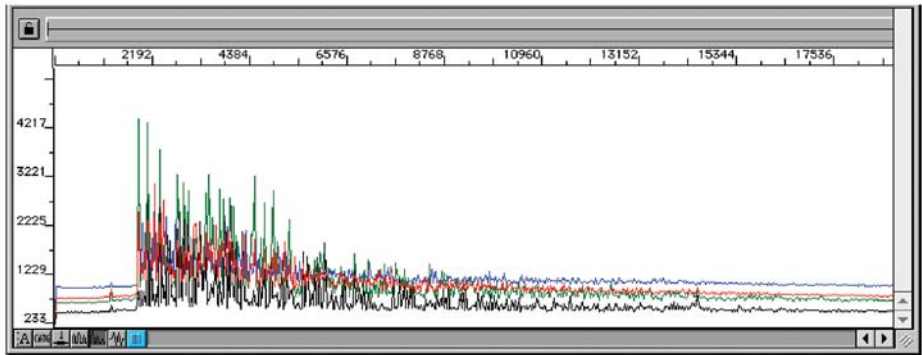


Fig. 5. Raw data from sequence failure resulting from salt contamination. Notice that the signal intensity commences strongly but quickly declines.

5. Sometimes the strongest peaks within a sequencing reaction have characteristic pull-up peaks beneath them (**Fig. 8A**). This is because of a phenomenon known as spectral overload. The fluorescent signal from the sequencing reaction is too great, and the matrix corrections to compensate for spectral overlap between the emission spectra of the different fluorescent dyes no longer function properly. If you have observed this phenomenon, check the raw data file for the sample. If some peaks in the raw data exceed approx 6000 fluorescent units, this is most likely to be the problem (**Fig. 8B**). Simply re-loading less of the sequencing product will correct this problem. Alternatively, carry out the sequencing reaction again, but add less template DNA. The symptoms of spectral overload may be easily confused with that of spectral overlap. See **step 6** in the troubleshooting guide.
6. When secondary peaks corresponding to a different dye color are observed under all peaks, regardless of the absolute value of the peak, spectral overlap is the cause



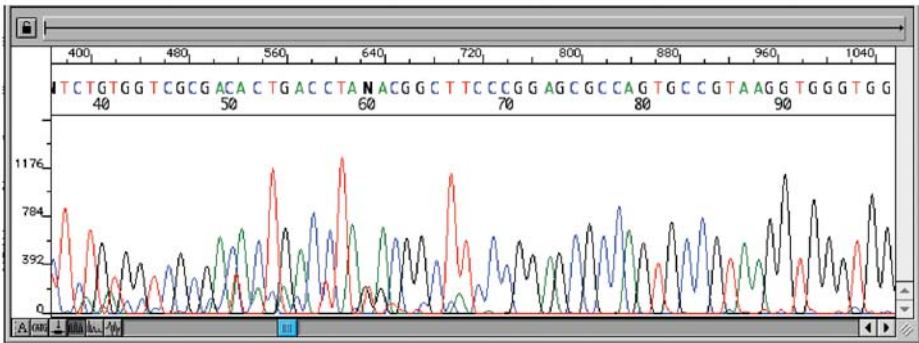


Fig. 6. Primer dimer contamination of the template. Note that the sequence is initially of poor quality, but after 67nt, suddenly exhibits good quality.

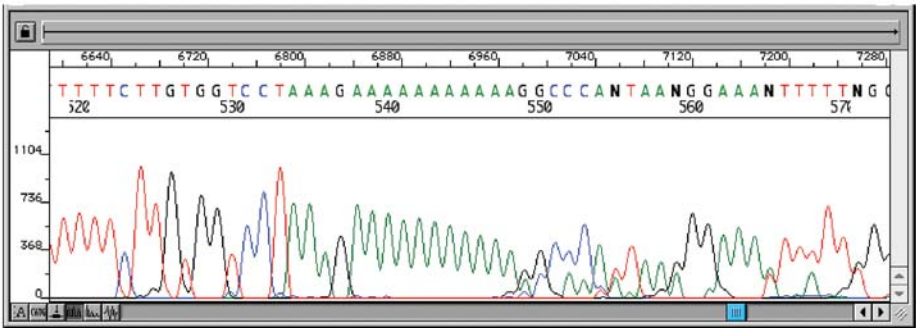


Fig. 7. Slippage and corruption of sequence data by a polyadenine repeat of 11nt.

(Fig. 9). This is caused by the matrix/spectral corrections not correctly compensating for the overlap in emission spectra between the different fluorescent dyes. There can be many reasons why this has occurred—e.g., use of a different electrophoresis polymer than that on which the original matrix/spectral file was calibrated, misalignment of the CCD camera caused by movement of the sequencer, or use of a different sequencing chemistry. Regardless of the underlying cause, the best remedy is to create a new matrix/spectral calibration file. Do this by re-running a set of matrix/spectral standards. Full details on this can be found within the user manual supplied with the sequencer.

### 3.9. Internal Quality Control

Internal quality control is extremely important in the context of diagnostic work. The following items are only recommendations, and should be viewed as a minimum set of standards for diagnostic sequencing. Furthermore, they

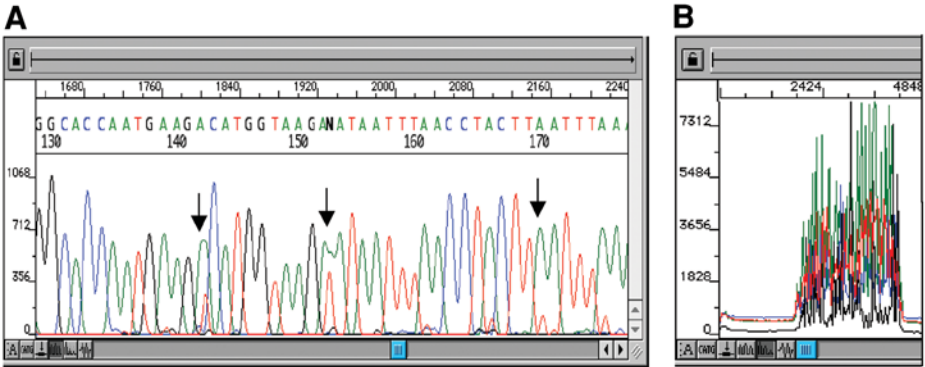


Fig. 8. Spectral overload. (A) Note the red “pull up” peaks under some of the larger green peaks (arrow). This is a result of the overall signal strength being too high and saturation of the matrix compensation. (B) When the raw data for this sequencing reaction is inspected, the signal from the green dye is particularly strong and many of the peaks exceed 6000 fluorescent units.

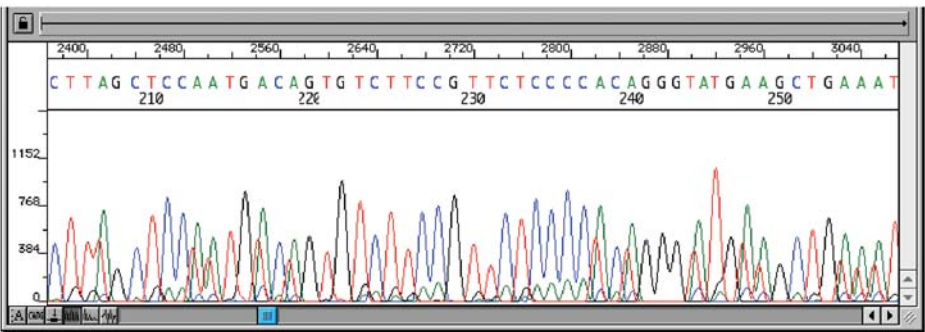


Fig. 9. Spectral overlap. Note the presence of minor peaks of consistent color beneath peaks from another dye (e.g., minor green peaks are consistently observed beneath blue peaks in the illustration).

only apply to the process of sequencing. Quality control should be operative at levels and all processes within a diagnostic laboratory.

1. When PCR-amplifying samples for sequencing, a negative or no DNA control should always be prepared. All samples should be monitored for amplification by agarose gel electrophoresis, and the negative control should remain clear of amplification. It is not considered necessary to carry through the negative control to sequencing.
2. Control should be exercised over sample transfers throughout the sequencing process.
3. When carrying out sequencing to define a previously uncharacterized mutation, the mutation should be clearly visualized on both strands i.e., in both orientations.

4. When this is not possible—for instance, because of proximity to primer or difficulties because of sequence context, a putative mutation identified in one orientation should be confirmed using another means, such as restriction digestion or mutation-specific PCR.
5. When using sequencing to test for a previously characterized mutation, unidirectional sequencing is adequate provided a positive and normal control are analysed simultaneously.
6. When using sequencing as a first-line mutation scan—e.g., in the absence of a pre-screen—any mutation observed should be confirmed on an independent PCR amplification either by sequencing or using an alternative technique.
7. All diagnostic sequencing data should be checked for accuracy and quality by an independent scientist prior to reporting.

#### 4. Notes

1. Try to minimize the length of time the gel slice remains on the transilluminator. Excessive exposure leads to nicking of the DNA backbone, degrading the DNA template and leading to truncation of the sequencing read.
2. Care must be taken when resuspending the SX resin in the Nucleon QC kits. The resin settles rapidly and must be resuspended by gently rolling or inverting either by hand or on a rotor. **Caution: Never** resuspend by shaking or vortexing, as this damages the resin and will adversely affect recovery yields.
3. When recovering precipitated DNA spun down in a fixed angle benchtop centrifuge, it is useful to orient the tubes in the rotor either by turning the tubes so that the tabs all face outward or by marking the exterior side of the lid with a marker pen. After centrifugation, the wall of the tube on which the DNA will be attached can then be identified so that the DNA pellet can be avoided when aspirating the supernatant.
4. Using sequencing diluent always leads to loss of signal strength, and with poorer quality templates this can turn what would have been an adequate reaction into an unsatisfactory one. Clearly, this can lead to unnecessary and expensive repeat assays. Sequencing diluent should only be used to save cost on properly validated templates that are known to sequence efficiently.
5. It can be difficult to accurately quantify the small volumes and low concentrations of sequencing template. A simple way to estimate the concentration is by ethidium bromide staining. Simply run a volume of purified template (e.g., 4  $\mu$ L) on a 1% agarose gel alongside a plasmid template of known quantity. The concentration of template can then be estimated by its relative fluorescence to the plasmid control.
6. With some sequencing primers that have high  $T_m$ s, greater specificity and thus better quality sequencing reactions can be obtained by increasing the annealing temperature above 50°C.
7. Multiscreen HV plates containing swollen G50 columns may be prepared in bulk and stored at 4°C in a sealed airtight container along with some dampened tissues. This maintains high humidity, thus preventing the columns from drying out.

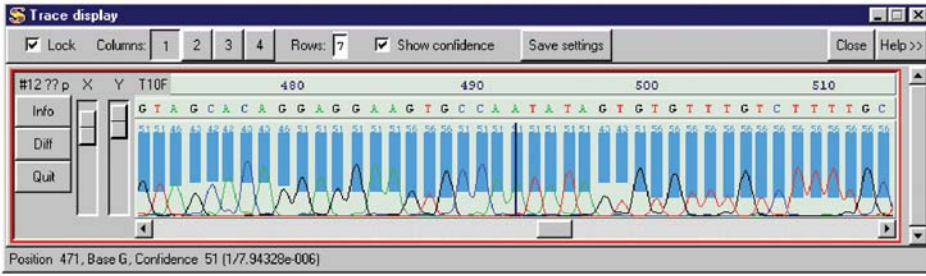


Fig. 10. Phred calculated base-calling accuracy indicated on the Gap4 trace display.

8. When carrying out mutation scanning by sequencing on slab gels, superior sequence data can be achieved by loading only alternate lanes. This prevents leakage between sample lanes during loading from interfering with and lowering the overall sequence quality.
9. The first time Gap4 is run, it defaults to *“Beginner”* mode. In order to carry out trace subtraction as described in **Subheading 3.6.3.**, Gap 4 must be set in *“Expert”* mode. Do this by selecting *“Configure Menus”* from the *“Options”* pull down menu. Gap4 can now be set into *“Expert”* mode from the dialog box that appears.
10. An alternative algorithm for displaying difference traces is available within Gap4. It can be selected by clicking the *“Settings”* button on the Contig Editor window and then selecting *“Trace display”* > *“Trace difference algorithm”* > *“Show positive differences”* from the series of menus. Some users prefer this mode of trace subtraction; however, it is still recommended to have *“Y scale differences selected”*.
11. The X and Y slider bars to the left of the trace display allows the user to alter both the relative peak heights and the amount of sequence displayed in the window. Any alterations to these settings are “remembered” for future displays of data.
12. If Phred was installed on the computer and the Phred module was selected as active when assembling the contig in Pregap4, clicking on the *“Show confidence”* box on the Gap4 trace display will display the confidence scores for each called base both as a histogram and as a numerical value (see **Fig. 10**). A Phred quality score of X corresponds to a base-calling accuracy of  $10^{-X/10}$ ; thus, a quality score of 20 indicates a base call of 99% accuracy.
13. You can also set Gap4 to Auto display traces and difference traces relative to a user determined control “normal” sequence. Set this option by clicking the *“Settings”* button on the Contig Editor window and then selecting *“Consensus trace options”* > *“Diff against a specific trace.”* Then enter the filename of the reference control sequence in the dialog box that appears.
14. When using trace subtraction to identify mutations, base-calling inconsistencies between samples, particularly at the beginning and end of the sequencing read, can give rise to artefacts on the difference trace (see **Fig. 11**). These can be easily resolved by cursory inspection of the electropherograms of the pair of sequences being compared.

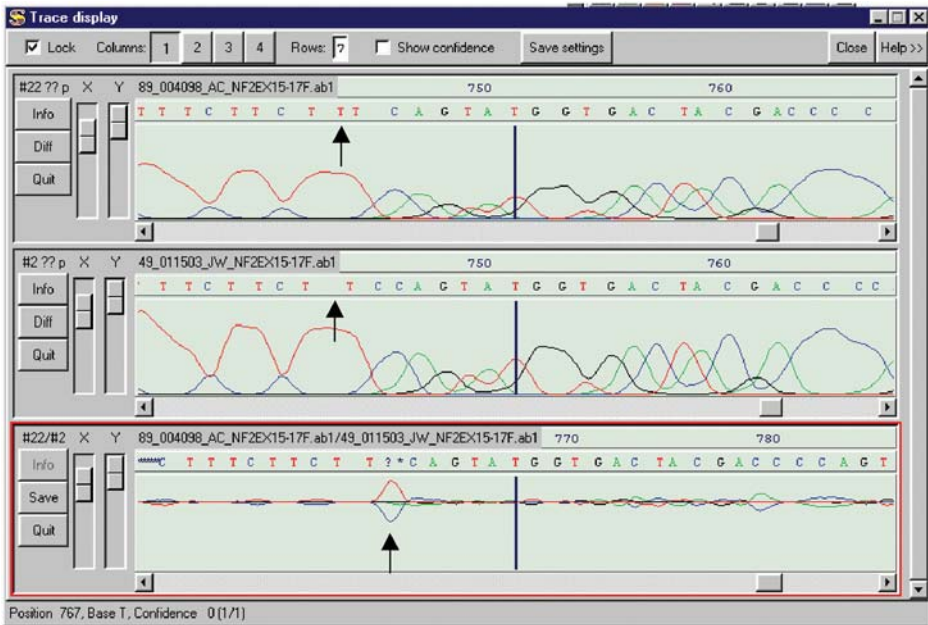


Fig. 11. Misalignment caused by base-calling errors leading to artefacts on the difference trace. A miscall of a run of 3 Ts as 2 Ts in the lower sequence (arrow) has led to misalignment.

15. Multiple sequence comparisons can be carried out simultaneously by double clicking on more sequences on the contig editor and creating more difference traces. The display settings may then be altered by clicking on the 2, 3, or 4 column buttons on the trace display window. However, displaying more traces in this way does reduce the window size.
16. From a study conducted of 215 single-heterozygote bases screened with Sequencer, five were incorrectly called as the original base with a secondary peak setting of 30% (2.3%). Although this figure could be further reduced by lowering the threshold, there is a danger of generating too many false-positives. Provided that fragments are sequenced in both forward and reverse orientations, the likelihood of the heterozygote base being missed—in theory at least—would seem to drop to insignificant levels.

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## Comparative Sequence Analysis

Chris Mattocks, Patrick Tarpey, and Jo Whittaker

### 1. Introduction

Automated fluorescent sequencing of polymerase chain reaction (PCR) products is now widely used in molecular diagnostics. It is most commonly used to characterize mutations detected in an initial screen using a less sensitive, indirect method, rather than a mutation scanning technique in its own right. This is largely because analysis software associated with automated DNA sequencing systems has been designed primarily to determine sequence, and not to determine the location and nature of heterozygous differences between sequences.

Typical sequence analysis software performs a large number of manipulations on raw fluorescence data, equalizing peak heights and spacing to facilitate accurate base-calling. There are software packages (e.g., Sequence navigator or SeqScape by Applied Biosystems [ABI]) that will align a number of sequences with respect to these base calls and then look for positions with two peaks, which may represent heterozygous mutations. It is not uncommon for these heterozygous peaks to drop out to the level of background noise and therefore be missed by the software. Sequencing for diagnostic purposes is generally performed in both directions, on the basis that any heterozygous mutations will be visible in at least one. In addition, it is often necessary to verify electropherograms by sight to ensure accurate results—a procedure that is laborious, time-consuming, and subject to error.

Comparative sequence analysis (CSA) is a simple alternative method for directly comparing base-lined raw data from equivalent sequencing reactions on different samples without the need for base calling (*1*). The control sequence electropherogram is split into its four component traces (one for each base), which are overlaid with equivalent traces from a second reaction. The result-



ing format allows for simple and rapid visualization of any differences (mutations) between the two sets of traces. Because the assay is based on sequencing, mutations can be characterized as well as identified. Moreover, because there has been minimal software manipulation of the data, heterozygous mutations that are difficult to pick up using standard sequencing analysis techniques have been shown to be easily detectable in a single direction using CSA. Finally—and perhaps most importantly from a diagnostic point of view—CSA is a simple and rapid technique for verifying normal sequence data with a very high degree of confidence.

## 2. Methods

The method described here has been specifically designed for analysis of data collected on the Perkin-Elmer ABI prism 377 DNA sequencer, although the principles may be transferable to other platforms (*see* **Notes 1** and **2**). Data collection software from version 2.0 up to 3.6 has been found to give equally satisfactory results. Use of the instrument and collection software is described in detail in the manufacturer's manual. Analysis is performed using ABI GeneScan software on a Macintosh computer (*see* **Note 2**). Again, no advantages have been identified that can be ascribed to a particular version. It is recommended that a general knowledge of the GeneScan software be acquired by reading the user's manual. The instructions in this chapter give a key-stroke guide to this straightforward computer-based process.

### 2.1. Sequencing Reactions and Electrophoresis

CSA is an analysis technique, and as such requires no deviation from standard sequence chemistry protocols. Techniques and chemistries are reviewed in detail elsewhere (**2,3**). Dye primer or dye terminator reactions work equally well. The following protocol is for use with BigDye terminators version 1.0 or 2.0 (*see* **Note 3**).

Sequencing and GeneScan gel files can both be opened in the GeneScan software for analysis using CSA. This means that archived sequence can be re-analyzed if required. However, once a Sequence gel has been opened in GeneScan, it cannot be re-opened in Sequence Analysis. Thus, the choice of collection software will depend on analysis requirements (*see* points 2 and 3).

1. Make a "CSA matrix" exactly as described in the GeneScan user's manual, but run the *sequence* matrix standards appropriate to the chemistry being used (*see* **Note 3**). This will only need to be done once for each chemistry used.
2. If only CSA is required, make a GeneScan sample sheet ("GeneScan sample") and run the gel as a "GeneScan run." This is simpler, as only a GeneScan sample sheet will be required. Use the run module appropriate to the sequencing and the "CSA matrix" (*see* **Subheading 2.1., step 1**).



3. If conventional sequence analysis is also required, make a sequence sample sheet (“Sequence sample”) and run the gel as a “Sequence run.” Use the module and instrument file appropriate for the chemistry used. Once the run is complete, locate and select the gel file (single click) and duplicate it by selecting “Duplicate” from the “File” menu. One gel file can now be used for sequence analysis, and the other for CSA. A new GeneScan sample sheet will need to be made for CSA (see **Subheading 2.2., step 3**).

## **2.2. Opening and Extracting a Gel**

1. Start the GeneScan program and open the “Gel file” by selecting “File” . . . “Open” . . . “Collection Gel.” It does not matter whether the gel has been run as a Sequence run or a GeneScan run.
2. If the gel has been run as a GeneScan run, **steps 3 and 4** will not be necessary.
3. Create and save a new GeneScan sample sheet by selecting “File” . . . “New” . . . “Sample sheet” (see **Note 4**).
4. Install the new sample sheet in the gel file by selecting “Gel” . . . “Install new Sample Sheet.”
5. Track and extract the gel as described in the GeneScan user’s manual.

## **2.3. Analyzing Extracted Files**

After extraction, the “Analysis Control” will appear on the screen containing files for all the extracted lanes. These do not always appear in the same order as listed in the sample sheet. This is not a problem, as the data is still associated with the correct name in the sample sheet. Samples can be sorted using “Sample File Sorting” under “Project Options” in the “Settings menu.” To arrange samples in the same order as the sample sheet, sort “As Added” under the first sorting preference.

### **2.3.1. Create a Size Standard**

CSA relies on being able to horizontally align equivalent traces from different sequencing reactions through the use of a size standard. The peaks in this standard are assigned size values, either true (reflecting the true size, in basepairs (bp) of the fragment that generated the peak) or arbitrary (in which case the only requirement is that the values increase with the true size of the fragments).

#### **2.3.1.1. EXTERNAL SIZE STANDARDS**

The best method for horizontal alignment is the use of an “external” size standard, which can be assigned true size values. This comprises a set of fluorescently labeled fragments that range in size from about 50 bp to 500 bp (or greater if required) in 50-bp increments. This type of standard is run in every lane of sequencing (by addition to the loading buffer), and is entirely distinct from the sequencing reaction.

Since the sequencing reaction itself requires four-color collection (one for each base), an external size standard must be labeled in a fifth color. In order to collect and analyze data comprising five colors, Collection software version 2.6 or above and GeneScan version 3.0 or above are required.

At the present time, ABI were able to supply a 500-bp Standard labeled with LIZ, which is suitable for this purpose. Alternatively, if greater size range is required, an “in house” size standard can be made by PCR amplification of a range of suitably sized fragments using fluorescently labeled primers or dUTPs, and mixing the products. A suitable dye for this purpose is Bodipy 630/650 (Molecular Probes, Leiden, the Netherlands).

Several points should be noted when using five-color collection:

1. The GeneScan sample sheet must be defined for use with the five-color collection. This choice is given when creating a new GeneScan sample sheet in version 3.0.
2. A new matrix is required to deal with the five colors. This should be made following the protocol described in the user’s manual using the matrix standards for the sequencing chemistry used together with the fifth dye.
3. After extraction in the analysis control, ensure that Orange is selected as the size standard. Define the size standard by opening the drop menu in the “Size Standard” column and selecting “Define New.” Assign appropriate sizes to the peaks in the size standard and save the file. This should only need to be done once for a particular size standard; the saved file can be used for all subsequent runs using the same size standard.
4. Apply the new size standard to all the files to be analyzed by selecting it from the drop down menu in the “Size Standard” column header or for individual files, the drop down menu in the size standard box relating to that file.

### 2.3.1.2. INTERNAL SIZE STANDARDS

The peaks representing one of the bases in the sequencing reaction can be used as an arbitrary size standard—this is known as an internal size standard and is technically the simplest method for horizontal alignment of traces. Because this type of standard is unique to the sequence being analyzed, the following procedure must be carried out for each different sequence run.

1. In the Analysis Control, select one of the colors to be the size standard by holding down the “apple” key and clicking the column header representing the chosen color.
2. Select one of the control lanes for analysis and click “Analyze.”
3. Open the analyzed file by double clicking the appropriate file name listed in the “Sample File” column. Ignore the “Cannot align by size” warning box: Click “OK.”

4. De-select the traces, which are *not* the chosen size standard color, by clicking on the appropriately colored boxes beneath the electropherogram.
5. Click the “Data Point” column header. This will highlight the whole column. Now copy the whole column by holding down the “apple” key and pressing “C” on the keyboard. Close the analyzed file.
6. Open the drop menu in the “Size Standard” column that relates to the analyzed file—select “Define New.”
7. In the table that appears below the electropherogram, select the top cell in the “Size” column. Now scroll to the bottom of the table and select the bottom cell while holding down the “Shift” key. This will select the whole of the “Size” column. Paste the size data into the column by pressing “V” while holding down the “apple” key. Check that the numbers in the “Data points” column match their partners in the “Size” column. Now close and save the new size standard (*see Note 5*).
8. Apply the new size standard to all the files to be analyzed by selecting it from the drop down menu in the “Size Standard” column header or for individual files, the drop down menu in the size standard box relating to that file.

### 2.3.2. Install a Matrix

Select all the files by clicking the “Sample file” column header. In the “Gel” menu, select “Install New Gel Matrix.” In the browse box, locate the desired matrix and click “OK.”

### 2.3.3. Analysis Parameters

It is important to note that traces with much lower intensities than will be analyzed by sequencing analysis can still be examined using CSA. Failure of a particular analysis is therefore user-defined, and it is important to set the analysis parameters correctly. Further guidance on evaluating data quality is provided in the notes section (*see Notes 6–9*).

Double click one of the <Analysis Parameters> in the right-hand column. In the options box that appears, check the following:

1. *Analysis Range*: ensure that this covers all data points on your gel.
2. *Data Processing*: make sure both the “Baseline” and “Multicomponent” options are ticked. The “Smooth Option” is mostly aesthetic, but can have an effect on the signal recognized GeneScan as a peak. For most applications, “Light” will work well.
3. *Peak Analysis Thresholds*: Should be set to at least 50 for each color. Peaks below this threshold are unreliable (*see Note 8*).
4. *Size Call Range*: select the “All Sizes” option.
5. *Size Calling Method*: This does not make much difference. In general, use “Local” or “Global” Southern.
6. *Split Peak Correction*: select “None.”



7. Click “OK” when ready.
8. Select all samples to be analyzed and Click “Analyze.”

## 2.4. Viewing CSA Traces

1. Select “Results Control” from the window menu.
2. Check that there are four panels and that panel 1 is selected. If using an external size standard with a five-color collection, there should be five panels. This will ensure that the traces appear in the panels in the order G at the top, and A, C, and T going down the screen (*see Note 3*). If panel 1 is not selected at this stage, this order will *not* be maintained, and confusion may arise.
3. Ensure “Quick Tile” is on.
4. It is simplest to view the traces so that all control traces are one color (e.g., black) and the test traces are a second color (e.g., red). Select “Custom Plot Colors” under “project Options” in the “Settings” menu. Check that color 1 is “Black,” and color 2 is “Red.”
5. Select “Results Display” under “Preferences” in the “Settings” menu. In the “Default Display Settings” select “Align By Size,” “Show Legends” and “Custom Plot Colors.”
6. Select the control sample by clicking on the appropriate numbered box on the left-hand side of the results control. With the “Quick Tile” engaged, this will automatically place the four colored traces in separate panels. Similarly, select the test sample to be compared with the control. It is important to select them in this order so that the control traces always appear black and the test red. Now click “Display.”

## 2.5. Navigation

The following is an overview of the “Display” window and its features. Notes refer to **Fig. 1**.

1. *Horizontal scale*: defines which region of the traces is being viewed—if you hold the cursor over this area, it changes to a . Double clicking the mouse now opens a dialog box in which you can change the horizontal scale. However, an easier way to zoom in and out is to hold down “apple” and press + (zoom in) or – (zoom out).
2. *Vertical scale*: (one for each panel) all four of these work the same as the horizontal scale (double click when the cursor changes to a .
3. *Dye color indicator*: shows the true color of the traces represented by the peaks in the panel above (*see Note 10*).
4. *Plot color indicator*: this, in conjunction with the *sample identifier*, shows which sample is represented by which colored trace in the panel above. In the example here, the black traces represent a “Normal female” and the red traces represent a “Normal male” (*see Note 11*).
5. *Scroll bar*: enables you to scroll along the traces when you have zoomed in.
6. *Scaling traces (vertical alignment)*: Vertical alignment of traces is important to enable accurate comparison of control and test samples. To alter the height of peaks

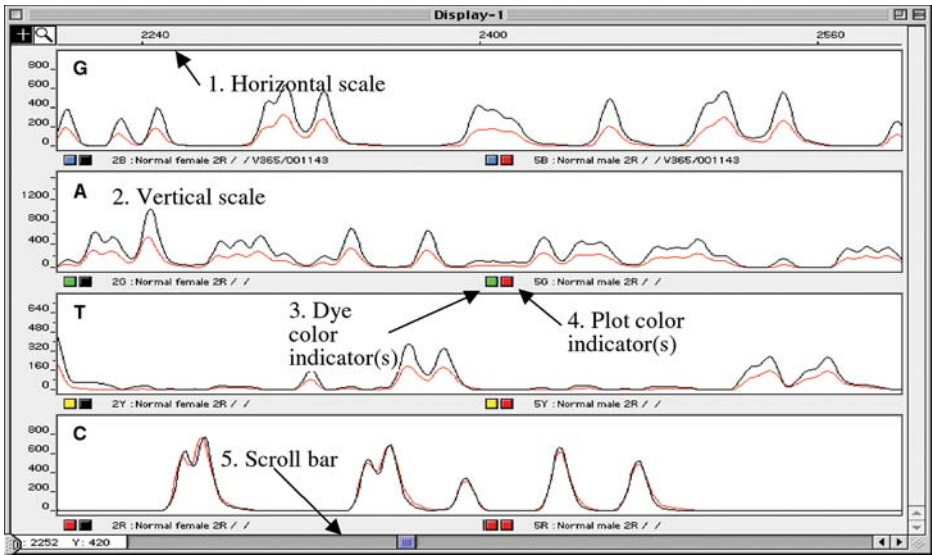


Fig. 1. The display window.

in a particular trace, double click the appropriate dye color indicator. This opens a dialog box. Entering 2 in the box doubles the height of the peaks in that trace, whereas entering 0.5 will halve the height of the peaks—any number can be entered in this box. The peaks of the RED trace (normal male) in the bottom panel have been scaled by a factor of 2. This has aligned the RED (normal male) and BLACK (normal female) traces on top of one another.

7. *To view another sample:* Close the current “Display window”—this will return you to the “Results Control.” Click “Clear All” to remove your previous selection and select a new pair of samples, as previously described.

## 2.6. Interpretation

Mutations are always characterized by two indicators (*see Note 12*).

1. For heterozygous mutations a “half height” peak indicates the “lost” or mutated base, and a “lone” peak at the same horizontal position indicates the mutant base (**Fig. 2A**).
2. For homozygous or hemizygous mutations, the mutated base will be entirely lost in the test sample.
3. Frame shift mutations are characterized by the same two indicators at the position of the mutation, and all downstream bases (to the right) will be offset by the number of bases involved in the mutation; to the left for deletions, and to the right for insertions (**Fig. 2B**).

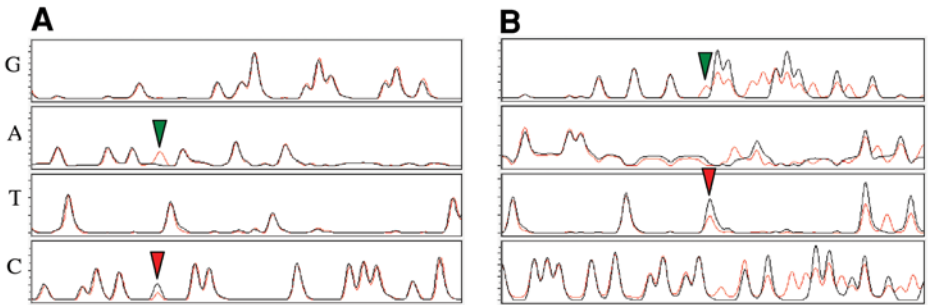


Fig. 2. (A) Heterozygous point mutation (C > A), (B) frame shift mutation (1 bp deletion).

4. Complex insertion/deletions will appear similar to a frame shift, but no particular offset pattern will be observable for the length of the insertion.
5. The position of a mutation can be determined by reading the sequence around the mutation and matching this with the known normal sequence.
6. If an external (fifth color) size standard is being used, the location of the mutation can be determined by clicking on the mutant peak. This will highlight the relevant data for that peak, which includes the size as determined by the size standard. This will give the relative position within the amplicon at which the mutation occurs.

### 3. Notes

1. CSA can be used for analysis of data collected on ABI capillary machines. In this case there is no "Gel File," so it is easiest to do a GeneScan run using the appropriate matrix. If a Sequencing run is used, the files can be converted into GeneScan format using an applet available free of charge from ABI. The new PC-based GeneScan package has slightly different "Analysis Parameters," but essentially the settings will be the same. However, making an internal size standard can be problematic, as the software tends to split one peak into several. This can be overcome with careful adjustment of the analysis parameters, particularly those dealing with peak definition. Once this is done, the setting should be generally applicable to subsequent runs. Alternatively an external size standard can be used or files can be converted to Mac format using a second applet also available from ABI, and analysis can be carried out in the normal way on a Macintosh computer.
2. Other systems such as the Base Station from MJ research (Cartographer software) can output data in ABI format. This should also be amenable to analysis in the normal way. Although CSA has not been attempted using software other than GeneScan, other systems (e.g., Cartographer) work in much the same way, and equivalent results may be attainable.
3. When dealing with dyes and matrices in GeneScan, it is important to note that the colors used to represent the different bases in Sequencing (G: yellow or black; C: blue; A: green; T: red) are artificial and dependent on the filter set. For example,

the actual colors of the dyes attached to BigDye version 1.0 terminators are G: blue; C: red; A: green; and T: yellow using the correct filter set (E). Therefore, if a different chemistry is being used, it is important to establish which dyes are attached to which bases and what color they are.

4. Sequencing sample sheets have one row per sample, whereas GeneScan sample sheets have four. This means that if a sequence sample sheet is used in GeneScan, data will not be attached to the correct sample label.
5. Essentially, the scan numbers are assigned to the peaks as their arbitrary size.
6. No mobility adjustments are made with CSA. Thus, bases will not necessarily be evenly spaced. This is most noticeable with G bases, which show retarded migration with respect to the other bases. A G following an A can appear to be almost in the same position, particularly at the beginning of the sequence.
7. The data being analyzed is still *Sequence Data*. This means that, except in the case of heterozygous mutations, there can only be one peak at any one position (considering the mobility of G bases). The clearest indicator of poor data is too many peaks. A simple way to assess the quality of the data is to view all traces together by double clicking on the sample name in either “analysis control” or “results control.” This displays an electropherogram similar to a standard sequence trace, but with no mobility adjustments.
8. The height of peaks (intensity) can be important. If intensity is too low, the peaks are called unreliably, and background is more significant. The “Peak analysis thresholds” set the minimum intensity at which GeneScan will recognize a peak. Clicking on the “Dye/Sample peak” column header in a sample display will highlight all the peaks above this threshold. This method can be used to check the intensity of a particular lane.
9. Lane-to-lane leakage on gels with staggered loading can be identified by opening adjacent lanes and aligning by scan. Leakage will be obvious as coincident peaks of the same color in both lanes.
10. All dye color indicators for a single panel are the same color. If GeneScan is set up as described in the text, the top panel represents the BLUE traces (or G bases), the next the GREEN traces (A bases), the next the YELLOW traces (T bases), and the bottom represents the RED traces (C bases).
11. If GeneScan is set up as described in the text, the first sample selected in the results control will be shown in BLACK and the second sample will be shown in RED. It is simplest to always use one color (e.g., BLACK) to represent the “normal,” leaving a second (e.g., RED) for the “test” sample.
12. The peak following a heterozygous point mutation often shows altered height. This is because of context-dependent differences in incorporation rates of the different dye terminators, and can be a useful third indicator of a mutation.

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## Gene Dosage Analysis by Multiplex Amplifiable Probe Hybridization

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### 1. Introduction

#### 1.1. Copy Number and Its Measurement

DNA-based measurement of copy number can be useful in a number of applications in human genetics, through its detection of rearrangements of chromosomal structure too small to be detected by standard cytogenetic analyses. In some cases, deletions or duplications may involve several linked genes (such as subtelomeric deletions and unbalanced translocations in idiopathic mental retardation [1–3]), and in others individual genes may have undergone duplications or deletions of the entire gene—for example, of *PMP22* in HSMN and HNPP (4)—or of exons within a gene—for example, in *DMD* (5) or *BRCA1* (6,7). The complete analysis of subtelomeric deletions and the analysis of exonic copy number of genes such as *BRCA1* (23 exons) requires the parallel measurement of copy number at more than 20 loci. Multiplex amplifiable probe hybridization (MAPH) provides a high-resolution assay for relative copy number at numerous loci (currently up to 60) in a single test (8).

#### 1.2. MAPH: How It Works

MAPH works on the principle that probes from different loci should hybridize to genomic DNA in amounts that are proportional to the copy number of their binding sites. In principle, if the amounts of different probes hybridized to a target DNA are individually quantified, this will allow the measurement of relative copy number. In MAPH, probes hybridized to genomic DNA are

```

      Blocker primer PZAX
      AGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGAT

      primer PZA
      AGTAACGGCCGCCAGTGTGCTG

      GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGat|atcCATCACACTGGCGGCCGCTCGAGCA
      +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
      CCTAGGTGATCATTGCCGGCGGTCACACGACCTTAAGACGTcta|tagGTAGTGTGACCGCCGGCGAGCTCGT
                                          GTAGTGTGACCGCCGGCGAGC
                                          primer PZB

                                          TAGGTAGTGTGACCGCCGGCGAGC
                                          Blocker primer PZBX
  
```

Fig. 1. The region of the pZero2 cloning vector (Invitrogen) around the *EcoRV* site. Blunt-ended fragments can be cloned into this site, and probes amplified using primers PZA and PZB. PZA and PZB can then be used as the primer pair to reamplify such probes after hybridization to genomic DNA.

detected and quantified by polymerase chain reaction (PCR) amplification of the bound probes. The resulting PCR products are distinguished by length after gel electrophoresis. The difficulties caused by multiplex PCR are avoided by configuring the probes so that all of them can be amplified using a single primer pair—thus “Amplifiable Probes.” The simplest way to create a set of probes with different inserts all flanked by the same primer binding site is to clone them into the same site of the same vector. Probe DNA is released from the plasmid by PCR using flanking primers derived from the vector. Most of our work to date has used inserts cloned into the *EcoRV* site of pZero2 (Invitrogen; see **Fig. 1** and **Note 1**), with probes created by amplification using flanking primers PZA and PZB.

For a copy number assay (**Fig. 2**), a set of probes—with different targets distinguished by length, but all flanked by the same primer binding sites—is denatured and allowed to hybridize to the test DNA, which has been denatured and immobilized on a filter. After hybridization has been allowed to proceed to near-completion, stringent washing is used to remove probes that are not specifically bound, leaving only probes that have found their respective target in the test DNA. These probes are then amplified using the flanking primers, under conditions in which the yield of product remains quantitative (e.g., proportional to the amount present at the start of the reaction). Separation of PCR products on a denaturing polyacrylamide gel then allows the products from different probes to be individually quantified. The relative intensity of these bands is the measure of relative copy number in the test DNA (8).

Some unusual features of this method are worth noting. First, the PCR products made in testing a genomic DNA sample are not amplified from that

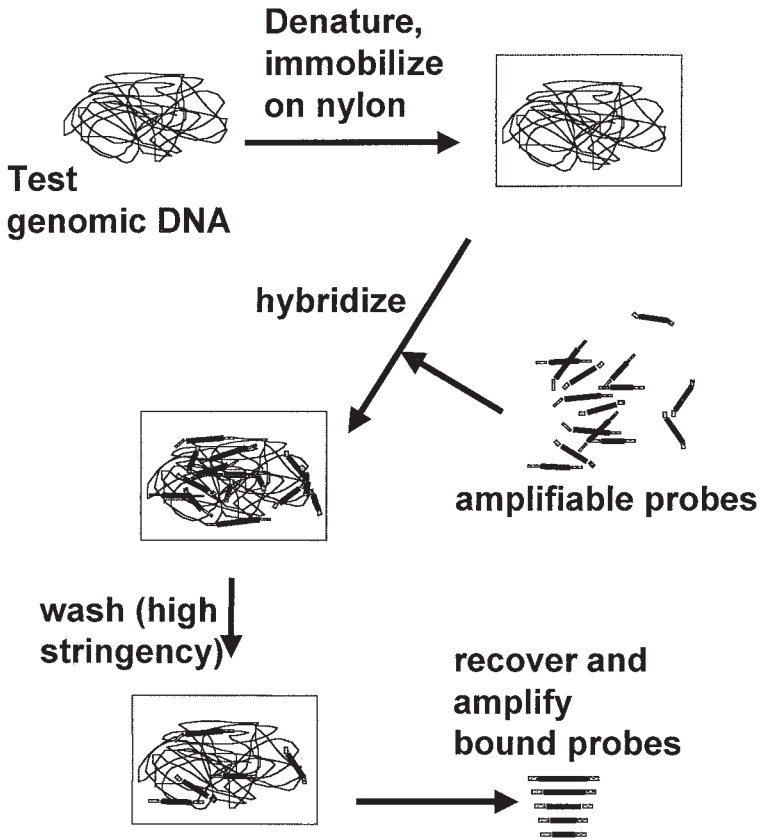


Fig. 2. General scheme for multiplex amplifiable probe hybridization.

sample—they are amplified from the *probes* added in the test, and the role of the genomic DNA is to select probes in amounts proportional to the copy number of their targets. Secondly, the probes used are relatively long (generally in the range 100–400 bp), so that substitutional variation in the sequence of the target site will not materially affect the amount of probe bound, and thus (unlike methods that use PCR from genomic DNA) MAPH should be unaffected by unexpected sequence polymorphism at the target site. Finally, all our work to date has used gel separation as a convenient method to distinguish and quantify amplified probes, which places a limitation on the number of probes that can be analyzed simultaneously. In principle, it is possible to quantify amplified products by hybridization back to an array, to allow the analysis of hundreds of probes in a single test.

In this guide, we will describe the use of MAPH to analyze copy number, including information on its implementation in fluorescent gel detection systems (such as Applied Biosystems [ABI] and ALF), and on the analysis of results.

## 2. Materials

### 2.1. Main MAPH Procedure

1. Genomic DNA: This must be at a relatively high concentration ( $>0.1$  mg/mL) since approx 1  $\mu$ g must be spotted, 1  $\mu$ L at a time, onto a membrane.
2. Nylon membrane: all our studies have used MAGNA membrane (OSMONICS, Westborough, MA: available from GRI [Braintree, Essex, UK]).
3. 1 M NaOH, reserved for denaturation of genomic DNA.
4. PCR equipment, such as PCR machine PE 9600 (Perkin-Elmer, Foster City, CA), containment facilities/reserved pipets.
5. Ultraviolet (UV) crosslinker—e.g., Gene Linker from Bio-Rad (Hercules, CA).
6. Prehybridization solution (0.5 M sodium phosphate pH 7.2, 7% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 100  $\mu$ g/mL alkali-denatured herring sperm DNA (Sigma-Aldrich, St. Louis, MO).
7. MAPH probe set (*see Note 2*).
8. Competitor DNAs: Human Cot-1 DNA (1 mg/mL, InVitrogen, Groningen, the Netherlands), *E. coli/HaeIII* DNA\* (at 3.5 mg/mL),  $\Phi$ X174/*HaeIII* (at 250  $\mu$ g/mL, ABgene, Epsom, UK), blocker primer mix (20  $\mu$ M each blocker primers PZAX and PZBX for probes cloned into the *EcoRV* site of pZero2). \**E. coli* DNA cut with *HaeIII*: digest 500  $\mu$ g *E. coli* chromosomal DNA with 50 U *HaeIII*, in a total of 0.5 mL 1X ReAct buffer 2 (InVitrogen). Phenol-extract, ethanol-precipitate, and redissolve in 50  $\mu$ L H<sub>2</sub>O. Check concentration by spectrophotometry.
9. 1 M NaOH and 1 M NaH<sub>2</sub>PO<sub>4</sub> (*see Note 3*).
10. Hybridization oven or 65°C water bath.
11. Washing solutions: (a) 1X standard saline citrate (SSC), 1% SDS; (b) 0.1X SSC, 0.1% SDS (500 mL of each, prewarmed to 65°C).
12. PCR reagents: 25 mM MgCl<sub>2</sub>, 25 mM deoxynucleotide 5' triphosphate (dNTPs), 10X PCR buffer (ABgene), reaction buffer IV: 10X buffer is 750 mM Tris-HCl, pH 8.8, 200 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween, *Taq* DNA polymerase (5 U/ $\mu$ L, we have used the enzyme from ABgene).
13. Polymerase chain reaction (PCR) primers (PZA/PZB for probes cloned into the *EcoRV* site of pZero2, each at 10  $\mu$ M).

Primer sequences (*see also Fig. 1*):

PZA 5' AGTAACGGCCGCCAGTGTGCTG 3'

PZB 5' CGAGCGGCCGCCAGTGTGATG 3'

PZAX 5' AGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGAT 3'

PZBX 5' CGAGCGGCCGCCAGTGTGATGGAT 3'

## 2.2. Radioisotopic Detection (Additional Materials)

1.  $\gamma$ -[<sup>32</sup>P] adenosine triphosphate (ATP) (or  $\gamma$ -[<sup>33</sup>P] ATP), T4 polynucleotide kinase (10 U/ $\mu$ L, InVitrogen), 5X T4 kinase “forward” reaction buffer (InVitrogen).
2. 0.5X TBE buffer (make as 10X TBE stock: 0.9 M Tris-base, 0.9 M boric acid, 0.02 M disodium EDTA).
3. Acrylamide mix (6% acrylamide/*bis*-acrylamide (19:1, Accugel, National Diagnostics, Atlanta, GA), 0.5X TBE, 50% w/v urea).
4. Formamide-loading mix (98% formamide, 10 mM EDTA pH 8.0, 1 mg/mL xylene cyanol FF, 1 mg/mL bromophenol blue).
5. Gel-fixing solution: 10% methanol/10% acetic acid.
6. Electrophoresis equipment: sequencing gel rig, high-voltage power pack, gel dryer.
7. Phosphorimager plus matching cassettes (Molecular Dynamics, Sunnyvale, CA).

## 2.3. Fluorescent Detection (for Use with ABI Sequencers) (Additional Materials)

1. PZA primer labeled with FAM (MWG-Biotech, Ebersberg, Germany), 10  $\mu$ M.
2. ROX-labeled markers (e.g., genSIZE T/500 from Genpak, New Milton, UK).
3. Loading buffer: 10 parts deionized formamide to one part 25 mM EDTA (pH 8.0), 50 mg/mL blue dextran.
4. ABI gel system (ABI, Foster City, CA).

## 2.4. Fluorescent Detection (ALF) (Additional Materials)

1. Cy5 labeled PZA primer (MWG-Biotech), 10  $\mu$ M.
2. ALF gel system (Amersham Pharmacia Biotech, Uppsala, Sweden).

## 3. Methods

### 3.1. Main MAPH Procedure

#### 3.1.1. Making the Filters

1. The denatured test DNA is immobilized on very small pieces of nylon filter (approx 1.5 mm  $\times$  3 mm). Since many different DNA samples can be hybridized together at one time, mark the number assigned to the filter with a sharp pencil. Do this before dividing up into individual filters (*see Note 4*).
2. Cut the filters from the nylon membrane (*see Note 5*), and arrange on a sheet of plastic food wrap film on the bench, with the pencil-marked surface down. Mark the number of each filter next to it on the plastic food wrap film.
3. Add 1  $\mu$ L of 1 M NaOH to about 1  $\mu$ g each genomic DNA sample (plus one H<sub>2</sub>O-only control). Spot this denatured DNA onto the filter, 1  $\mu$ L at a time, allowing the filter to dry between applications (**Fig. 3A**).
4. When all the DNA has been applied to the filters, allow them to dry and wrap them up in the food wrap.

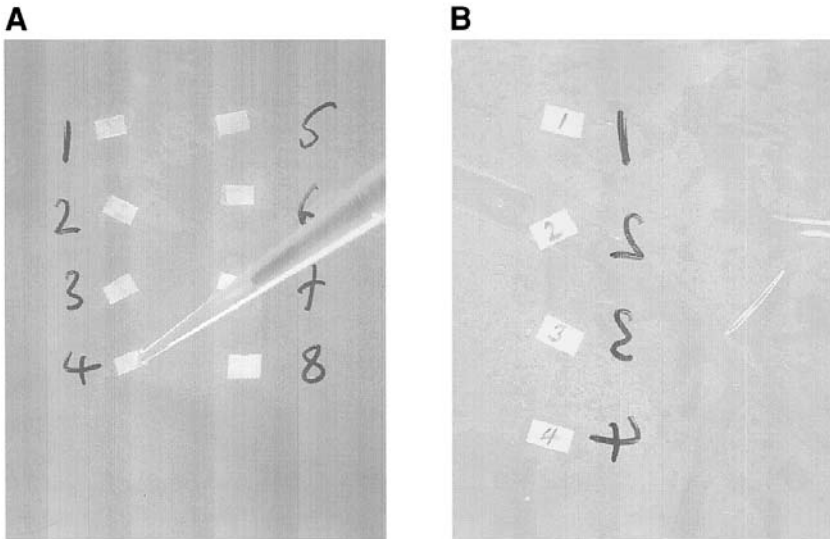


Fig. 3. (A) Spotting DNA onto the filters. Note that the pencil-labeled side is facing down, and the DNA (1  $\mu\text{L}$  at a time) is added to the upper surface. Numbers are marked next to each filter on a sheet of clingfilm in which the filters are subsequently enclosed for uv crosslinking. (B) After crosslinking, photograph the pencil-marked side of each filter, so that there is a record of the actual appearance of each filter's number.

5. Crosslink the DNA to the filters by uv irradiation (254 nm, 50–70 mJ, both sides).
6. (Optional) Take a photograph of the filters using a standard gel-documentation system, photographing the pencil-marked (e.g., non-DNA) side (**Fig. 3B**). This can be useful later if there is any doubt about the expected appearance of any one filter).

### 3.1.2. Prehybridization and Hybridization

As many as 24 filters can be hybridized together in the same tube. For larger numbers, set up a second hybridization.

1. Prehybridize the filters together in a screw-top microcentrifuge tube containing 1 mL prehybridization solution (0.5 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA, 100  $\mu\text{g}/\text{mL}$  alkali-denatured herring sperm DNA) at 65°C for 2–16 h.
2. Add 3  $\mu\text{L}$  human Cot-1 DNA (1 mg/mL) to 300  $\mu\text{L}$  of fresh prehybridization solution in a screw-top tube, and incubate at 100°C for 2 min.
3. Remove the prehybridization solution from the filters and replace with 200  $\mu\text{L}$  of the solution boiled in **step 2**. Incubate at 65°C for 30–60 min.
4. Add 1  $\mu\text{L}$  Cot-1 DNA (1 mg/mL), 2  $\mu\text{L}$  *E. coli*/HaeIII DNA (at 3.5 mg/mL), 2  $\mu\text{L}$   $\Phi\text{X174}/\text{HaeIII}$  (at 250  $\mu\text{g}/\text{mL}$ ) and 1  $\mu\text{L}$  blocker mix (*see Note 6*) to 0.5–1  $\mu\text{L}$  of

probe mix. Add 2  $\mu\text{L}$  of 1 M NaOH to denature the probe and incubate at 37°C for 1 min. Then place on ice and add 3  $\mu\text{L}$  of 1 M  $\text{NaH}_2\text{PO}_4$ ; mix, to neutralize the alkali, and add to the hybridization mixture from **step 3**. Incubate at 65°C overnight (see **Note 7**).

### 3.1.3. Posthybridization Washes and PCR

1. Pipet off the hybridization mix, and replace with 1 mL prehybridization solution at 65°C: mix and remove from the filters. Transfer the filters into a 50-mL centrifuge tube by rinsing them out with 1 mL of prewarmed 1X SSC, 1% SDS.
2. Wash at 65°C in 1X SSC, 1% SDS (total of 500 mL, prewarmed to 65°C) followed by 500 mL of 0.1X SSC, 0.1% SDS, 65°C. Do 5–6 washes of 50 mL 1X SSC, 1% SDS (corresponding to full centrifuge tubes), and then place the tube in the oven's carousel and leave to rotate for 5 min. Do another 2–3 washes of 50 mL 1X SSC, 1% SDS and rotate for 5–10 min. Use the remaining 1X SSC, 1% SDS in batches of 50 mL, and continue the process of alternating 2–3 washes of 50 mL and rotation on the carousel but now using 0.1X SSC 0.1% SDS. In total, wash for 45–60 min, using all of solution (a) in the first 15–20 min (see **Note 8**).
3. When all washing solution is used up, tip out the filters into a Petri dish. Identify each filter before placing into its own PCR tube (see **step 4**); the first stage in recovering the probes specifically bound to the filter involves a 5-cycle, 50- $\mu\text{L}$  PCR using a PCR machine with a heated lid (e.g., no oil).

#### PCR (stage 1)

mix (per filter, plus one for PCR control):

a.	10X PCR mix	5 $\mu\text{L}$	
b.	25 mM $\text{MgCl}_2$	2 $\mu\text{L}$	
c.	10 $\mu\text{M}$ primer A	1 $\mu\text{L}$	[e.g., PZA]
d.	10 $\mu\text{M}$ primer B	1 $\mu\text{L}$	[e.g., PZB]
e.	25 mM dNTPs	0.4 $\mu\text{L}$	
f.	Taq (5 U/ $\mu\text{L}$ )	1 $\mu\text{L}$	
g.	$\text{H}_2\text{O}$	<u>39.6 <math>\mu\text{L}</math></u>	
		(~50 $\mu\text{L}$ )	

4. Dispense 50- $\mu\text{L}$  aliquots of the above mix into thin-walled 200- $\mu\text{L}$  PCR tubes, and add the corresponding filter, transferring as little as possible of the washing solution with it (see **Note 9**). The filter is simply placed into the PCR mix (**Fig. 4**); the specifically bound probes will be released during the first denaturation step and will become available for subsequent amplification.
5. Cycle at 95°C 1 min, 60°C 1 min, 70°C 1 min for five cycles only in a PCR machine with a heated lid, to allow oil-free reactions. This primary PCR can then be kept to seed numerous secondary PCRs using any one of the detection methods outlined in **Subheadings 3.2.–3.4.** (see **Note 10**).



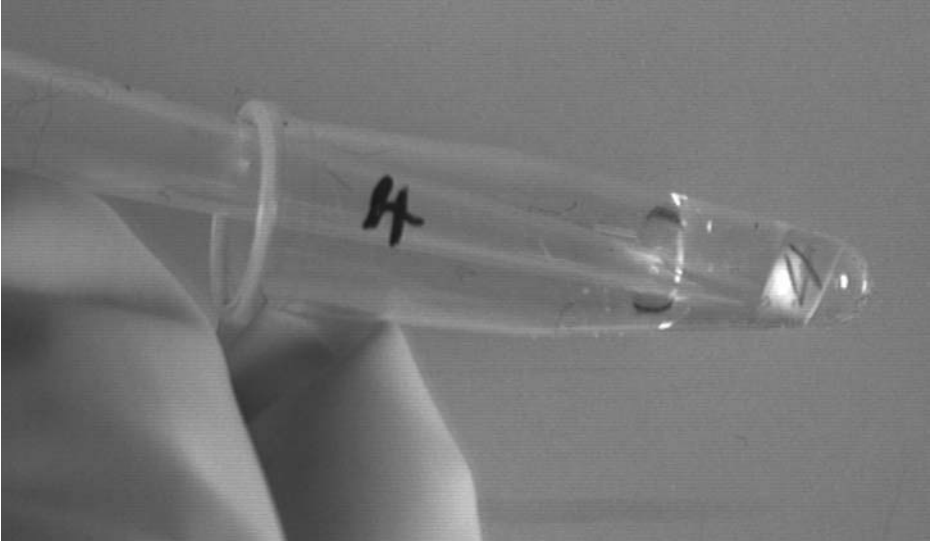


Fig. 4. Placing the washed filter in the (50  $\mu\text{L}$ ) primary PCR. Pipet tips, as shown here, can be used to manipulate the filter into the tube.

### 3.2. Radioisotopic Detection

1. End-label sufficient PZA primer, allowing 1  $\mu\text{L}$  per reaction. For example, for 10 reactions, mix:

a. 10 $\mu\text{M}$ PZA	2 $\mu\text{L}$
b. 5X forward reaction buffer	2 $\mu\text{L}$
c. T4 polynucleotide kinase (10 U)	1 $\mu\text{L}$
d. $\gamma$ -[ $^{32}\text{P}$ ] ATP	2 $\mu\text{L}$
e. $\text{H}_2\text{O}$	<u>3 <math>\mu\text{L}</math></u>
(total)	10 $\mu\text{L}$

incubate at 37°C, at least 2 h (overnight if convenient).

2. For radiolabeled PCR, use 1  $\mu\text{L}$  from the primary PCR, plus 9  $\mu\text{L}$  as follows:

a. 10X PCR mix	1 $\mu\text{L}$	
b. 25 mM $\text{MgCl}_2$	0.4 $\mu\text{L}$	
c. 25 mM dNTPs	0.08 $\mu\text{L}$	
d. labeled primer A	1 $\mu\text{L}$	( $^{33}\text{P}/^{32}\text{P}$ end-labeled, 2 $\mu\text{M}$ )
e. 10 $\mu\text{M}$ primer B	0.5 $\mu\text{L}$	
f. Taq (5 U/ $\mu\text{L}$ )	0.1 $\mu\text{L}$	
g. $\text{H}_2\text{O}$	<u>5.92 <math>\mu\text{L}</math></u>	
	(9 $\mu\text{L}$ )	

cycle 95°C 1 min/60°C 1 min/70°C 1 min, for 20 cycles, then hold at 72°C for 20 min—drives terminal dA addition to completion (*see Note 11*).

3. Add 10  $\mu\text{L}$  formamide loading mix to each sample. Denature at 100°C for 2 min and place on ice.
4. Run 4  $\mu\text{L}$  of each sample on a 6% denaturing polyacrylamide (sequencing) gel, until the xylene cyanol has just gone off the end of the gel.
5. Fix the gel in 10% methanol/10% acetic acid (10–20 min), dry and detect the radioactivity by exposure in a phosphorimager cassette.

### 3.3. Fluorescent Detection Using ABI 377 Prism Technology

1. Use 1  $\mu\text{L}$  from the primary PCR, plus 19  $\mu\text{L}$  of the mix, as follows:

a. 10X PCR buffer	2 $\mu\text{L}$
b. 25 mM $\text{MgCl}_2$	0.8 $\mu\text{L}$
c. 25 mM dNTPs	0.16 $\mu\text{L}$
d. 10 $\mu\text{M}$ 6-FAM-PZA	0.4 $\mu\text{L}$
e. 10 $\mu\text{M}$ PZB	0.4 $\mu\text{L}$
f. Taq DNA polymerase (5 U/ $\mu\text{L}$ )	0.4 $\mu\text{L}$
g. $\text{H}_2\text{O}$	14.84 $\mu\text{L}$

Cycle 95°C for 1 min, 60°C for 1 min, and 70°C 1 min, for 20 cycles, then hold at 72°C for 20 min.
2. The first method, which follows, should be suitable for most probe sets. However, for a large probe set (48 probes), we have found that concentration by ethanol-precipitation (the second method that follows) is necessary to achieve a sufficient signal on the gel.

Either:

Transfer 2  $\mu\text{L}$  of the PCR products to a 0.5-mL tube, and add 1  $\mu\text{L}$  of a 60:1 mixture of loading buffer and ROX-labeled size marker, and maintain at 4°C until the gel is ready to load.

Or:

Transfer the PCR products to a 0.5-mL tube, and add 20  $\mu\text{L}$  of 0.4 M NaCl and 100  $\mu\text{L}$  of ethanol, then mix by inversion. Incubate at room temperature for 15 min. Spin in a microfuge (10,000g) for 10 min, and remove the supernatant carefully, ensuring that the pellet is not disrupted. Leave to dry in air for at least 5 min until the pellet is *completely* dry.

Add 3  $\mu\text{L}$  of a 60:1 mixture of loading buffer and ROX-labeled size marker and keep at 4°C until the gel is ready to load.

3. Set up a 5% gel using Longranger gel mix (FMC Corporation, Rockland, ME) and 6 M urea. After prerunning the gel for 10 min, denature the sample at 95°C for 3 min, place on ice, then load 1.5  $\mu\text{L}$  on alternate odd-numbered wells. Prerun the gel for 3 min, then load the remaining samples on the even-numbered wells.
4. Run the gel for 3½ h.

### 3.4. Fluorescent Detection by ALFexpress

- |                                  |                               |
|----------------------------------|-------------------------------|
| 1. Secondary PCR:                |                               |
| a. 10X PCR mix                   | 1 $\mu$ L                     |
| b. 25 mM MgCl <sub>2</sub>       | 0.6 $\mu$ L                   |
| c. 25 mM dNTPs                   | 0.08 $\mu$ L                  |
| d. 5 $\mu$ M CY5-PZA             | 1 $\mu$ L                     |
| e. 10 $\mu$ M PZB                | 1 $\mu$ L                     |
| f. Taq polymerase (5 U/ $\mu$ L) | 0.1 $\mu$ L                   |
| g. H <sub>2</sub> O              | <u>5.22 <math>\mu</math>L</u> |
| h. (subtotal)                    | 9 $\mu$ L                     |
| i. Input (from primary PCR)      | <u>1 <math>\mu</math>L</u>    |
| j. Total                         | 10 $\mu$ L                    |

Cycle at 95°C 1 min/60°C 1 min/70°C 1 min, for 22 cycles, then hold at 72°C for 20 min.

- Add 8  $\mu$ L deionized-formamide loading buffer to 4  $\mu$ L of secondary PCR product, denature at 95°C for 5 min, then place on ice (*see Note 12*).
- Run all of the products on a denaturing polyacrylamide gel (SequaGel or High Resolution ReproGel, Amersham Pharmacia Biotech). The following conditions work well (*see Note 13*):
  - Voltage: 1500 V
  - Current: 60 mA
  - Power: 30 W
  - Temperature: 55°C
  - Sample interval: 2 s
  - Buffer: 0.5X TBE
  - Run time: 150 min (on short gel, and with largest amplicon of 550 bp).

## 4. Results and Analysis

Whichever method is used to detect the final PCR products, the results will be in the form of a series of bands (corresponding to the probes used), from which relative peak areas can be derived. **Figure 5** shows results from four DNA samples using a probe set including probes from the *PMP22* gene, while the more complex profile in **Fig. 6** uses a *BRCA1* exonic probe set with a series of ten genomic DNA samples, including two examples of an exon 13 duplication (arrow), detected using a Cy5-labeled primer in an ALF gel system. Although peak heights vary for each probe, and different samples yield different amounts of total signal, the method aims to measure the relative copy number of each probe. The *relative* intensity of any one band is thus the important measurement. Exactly how this is expressed numerically depends on the application. In applications such as screening of subtelomeric loci, in which it is expected that only one or two loci might be abnormal, it is sufficient to compare each peak with its neighbors. For screens of exonic probes, for which it is (at least

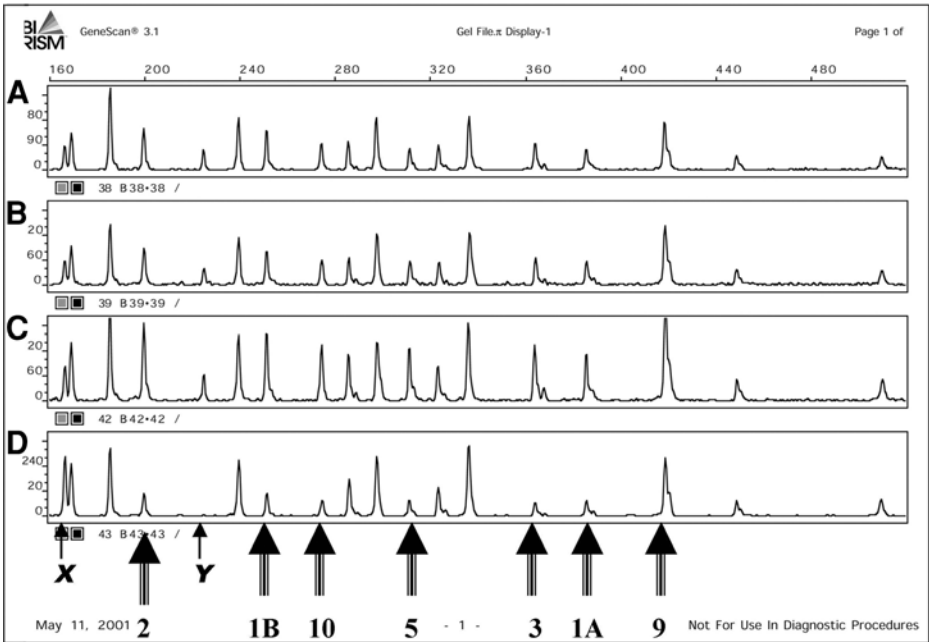


Fig. 5. Results of MAPH with a relatively simple probe set, consisting of probes from the *PMP22* gene (double arrows), sex-linked probes (single arrows), and autosomal control probes (unmarked). A and B are control males, C is a male with a duplication of *PMP22* (HMSN I), and D is a female with a deletion of *PMP22* (HNPP).

in principle) possible that all probes have undergone a parallel change—such as would occur in a whole-gene deletion—each probe can be compared with reference probes from other autosomal loci that are assumed not to vary in copy number.

For each series of probes for a given sample, each peak area is first converted to a fractional value relative to other peaks; in the example below (Tables 1–3), which is from part of the *BRCA1* probe set, each exonic peak is compared with the two nearest peaks.

The peak area for the probe for exon 13, for example, is expressed as a fraction relative to the nearest two probes (e.g., 24 and 21), so the fractional value for probe 13 is  $[13]/([21]+[24])$ .

This “fractional value” for that peak should be comparable between samples if there has been no copy number change. To convert to a “normalized ratio,” the mean fractional value is calculated for each probe (final column, in italics, in Table 2), allowing each result to be expressed in a scale in which 1.0 is the mean value among the samples tested. In this example, the peak areas for adja-



AlleleLinks  
bc7.#013 alk bc7a fmv

13 Apr 100 08 33.31

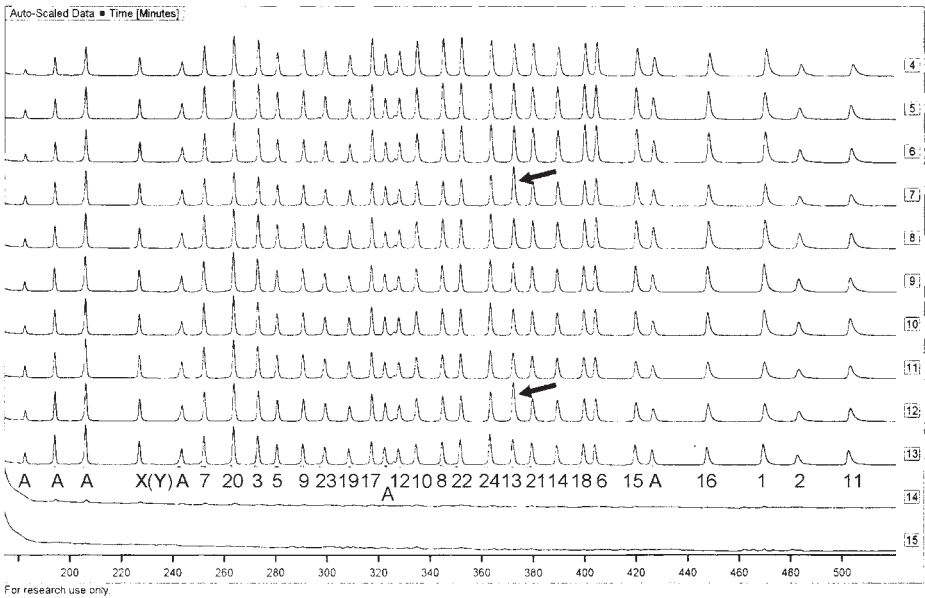


Fig. 6. Results of MAPH using a probe set consisting of the exons of *BRCA1* (numbered), as well as reference probes from other autosomal regions (A) and an X-linked probe (X). There is also a Y-linked probe (Y), but all these samples are from females. There are twelve results shown, from 10 women and two controls (the last two traces). Traces 4 and 9 show duplications of exon 13 (arrows).

cent probes are all roughly comparable, so the fractional values are approx 0.5. In **Table 3**, exon 13 appears as a duplication in the heterozygous state in samples 4 and 9, corresponding to a normalized ratio of approx 1.5 (boxed).

For a given probe set, evaluation of results can be made more robust by making use of prior experience with the same probes in unaffected control samples. The significance of putative abnormalities can thus be evaluated by calculating the number of standard deviations by which the observation departs from a mean value normalized to 1.

The use of MAPH as a diagnostic test requires caution. A new probe set should be tested against a series of control DNAs that are not expected to contain deletions or duplications, together with as many positive controls as possible. As MAPH works by hybridization, polymorphisms in homologous sequences can produce apparent changes in the copy number of the gene(s) being tested. The possibility of this occurrence can also be tested by computer

**Table 1**  
**Raw Data from Gel**

exon	sample									
	1	2	3	4	5	6	7	8	9	10
<b>8</b>	1152	2812	1575	1704	1026	1021	881	805	651	650
<b>22</b>	1041	2574	1560	1665	917	963	780	769	586	644
<b>24</b>	1143	2717	1820	2044	1168	1246	1086	970	747	867
<b>13</b>	1056	2896	1841	3315	1067	1145	920	922	1182	737
<b>21</b>	1050	2540	1716	1809	941	1043	852	805	597	641
<b>14</b>	1001	2687	1714	1833	989	965	810	765	608	601
<b>18</b>	1020	2723	1811	1727	980	983	843	722	578	579

**Table 2**  
**Exonic Signals (Fractional Values)**

exon	fractional value										mean
	sample										
	1	2	3	4	5	6	7	8	9	10	
<b>8</b>	0.552	0.552	0.539	0.501	0.555	0.545	0.550	0.522	0.531	0.521	0.537
<b>22</b>	0.454	0.466	0.459	0.444	0.418	0.425	0.397	0.433	0.419	0.425	0.434
<b>24</b>	0.521	0.504	0.581	0.607	0.601	0.628	0.654	0.616	0.604	0.670	0.599
<b>13</b>	0.482	0.551	0.521	0.860	0.506	0.500	0.475	0.519	0.879	0.489	0.578
<b>21</b>	0.520	0.470	0.487	0.508	0.478	0.535	0.515	0.541	0.503	0.543	0.510
<b>14</b>	0.484	0.511	0.486	0.518	0.515	0.476	0.478	0.501	0.517	0.493	0.498
<b>18</b>	0.508	0.519	0.533	0.473	0.497	0.516	0.523	0.492	0.506	0.507	0.507

**Table 3**  
**Normalized Ratios**

exon	normalized ratio									
	sample									
	1	2	3	4	5	6	7	8	9	10
<b>8</b>	1.029	1.028	1.004	0.934	1.035	1.015	1.024	0.973	0.989	0.970
<b>22</b>	1.045	1.073	1.059	1.024	0.963	0.979	0.914	0.998	0.966	0.978
<b>24</b>	0.871	0.843	0.970	1.014	1.004	1.049	1.092	1.030	1.009	1.119
<b>13</b>	0.833	0.953	0.900	1.488	0.875	0.865	0.821	0.898	1.521	0.845
<b>21</b>	1.019	0.920	0.954	0.996	0.937	1.050	1.011	1.061	0.987	1.065
<b>14</b>	0.971	1.025	0.976	1.041	1.034	0.957	0.960	1.006	1.039	0.989
<b>18</b>	1.002	1.023	1.051	0.933	0.979	1.016	1.030	0.969	0.997	1.000

matching against available genomic sequence when designing the probe set. A second problem encountered has been the occasional creation of false “fractional values” following problems with gel running. For these reasons, it is wise to confirm apparent duplications or deletions by repeating the test, followed by the use of alternative techniques.

## 5. Notes

1. pZero2 is a positive selection vector, which selects for recombinants by the interruption of a lethal gene. In principle, MAPH probes could be made by using 5' tailed amplimers, but using clones allows easier purification via isolation of single bacterial colonies.
2. Making probe sets is easy, but very tedious. It is hoped that sets of probes for popular applications will be made available commercially. For updates on probe availability, see <http://www.nott.ac.uk/~pdzjala/maph/probes.html>.
3. This stock of 1 M NaOH, which will come into contact with “neat” probe mix, should be a different stock from the (PCR-clean) one used to denature genomic DNA.
4. Make a strip of nylon of the right width, and then mark the numbers with a sharp 2B pencil before dividing into individual filters. It is much easier to write small numbers on a strip than on a single filter!
5. Turn the filters numbered side down on the piece of plastic food wrap film so that the DNA is spotted onto the unmarked side. PCR-clean facilities (such as a clean room) that do not involve cabinets with high velocity air currents are recommended for this step. A laminar flow cabinet is not suitable for such tiny filters. If necessary, denature the genomic DNA under PCR-clean conditions in the cabinet, but spot it onto the filters on the lab bench, using a reserved pipet.
6. The blocker primers PZAX and PZBX compete with “illegitimate” interactions between non-cognate probes resulting from hybridization between their common flanking segments. The other various DNA sources are intended to be general blocking agents to reduce nonspecific hybridization by the probes. We have not done any testing to determine which (if any) of these are really necessary.
7. When first adding the denatured probe, mix well by pipetting up and down. Once the probe has reached all parts of the hybridization solution, hybridization proceeds well without further agitation. It is possible that greater uniformity might be achieved by mixing throughout the hybridization, but this is not easy to arrange in a vol of 200  $\mu$ L.
8. Since up to 50 tiny filters are being washed at once, some vigilance is required to ensure that no filters are lost. This risk can be reduced by decanting each wash into a beaker, rather than directly into a sink, and by using a tea-strainer. The only fail-safe method is to count the filters carefully before discarding the contents of a rinse beaker.
9. At this stage, the input contains PCR products (the probes bound to the filters), but PCR contamination must be avoided. This is also true for the secondary PCR.



At this stage, make the master mix under PCR-clean conditions, make 50- $\mu$ L aliquots and then add the filters in the general lab.

10. Note that there are two negative controls per experiment. One is a filter without DNA, and the other is a PCR without input. If either of these shows evidence of contamination, its source can be narrowed down by comparison between the two.
11. These conditions appear to drive the addition of terminal dA to near-completion, so that the signal from each probe is represented in a single product, rather than pairs of peaks differing by 1 bp.
12. Deionized formamide: 1 g resin (homogenous mixture of cation and anion resin, Amberlite, Amersham Pharmacia Biotech) is added to 10 mL formamide and the mixture is shaken for 30 min and then centrifuged. The upper clear solution is collected and stored at  $-20^{\circ}\text{C}$ .
13. ReproGel High Resolution has a final concentration of 8%(w/v) acrylamide/bisacrylamide monomers and 1X TBE (0.09 M Tris, 0.09 M Boric acid, 1 mM EDTA). ReproGel High Resolution should only be used for ultraviolet (UV) polymerization together with ReproSet.

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## Prenatal Detection of Chromosome Aneuploidy by Quantitative Fluorescence-PCR

Kathy Mann

### 1. Introduction

Autosomal chromosome aneuploid pregnancies that survive to term—namely trisomies 13, 18, and 21—account for 89% of chromosome abnormalities with a severe phenotype (1). They are normally detected by full karyotype analysis of cultured cells. The average UK reporting time for a prenatal karyotype analysis is approx 14 d (2), and in recent years there has been an increasing demand for more rapid prenatal results with respect to the common chromosome aneuploidies, in order to relieve maternal anxiety and facilitate options in pregnancy. The rapid tests that have been developed negate the requirement for cultured cells, instead directly testing cells from the amniotic fluid (AF) or chorionic villus sample (CVS), with the aim of generating results within 48 h of sample receipt. Interphase fluorescence *in situ* hybridization (FISH) (3,4) is the method of choice in many genetic laboratories, usually because the expertise and equipment is readily available. However, a quantitative fluorescence-PCR (QF-PCR) based approach is more suited to a high-throughput diagnostic service. This approach has been investigated in a small number of pilot studies (5–7) and reported as a clinical diagnostic service (8). It may be used as a stand-alone test or an adjunct test to full karyotype analysis, which subsequently confirms the rapid result and scans for other chromosome abnormalities not detected by the QF-PCR assay.

### 2. Principle

QF-PCR refers to the amplification of chromosome-specific polymorphic microsatellite markers using fluorescence-labeled primers, followed by quantitative analysis of the products on a genetic analyzer to determine copy number

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and/or imbalances of specific chromosomal material. Tetranucleotide repeat markers are used to minimize PCR-generated “stutter bands” (amplified sequences that are 1–3 repeat units smaller than the true allele size). When a microsatellite marker is heterozygous, the ratio of its allele peak areas represents a disomic (1:1) or trisomic (2:1, 1:2 or 1:1:1) chromosome complement (*see Fig. 1*). A marker is uninformative if only a single peak is observed.

Because of allele size heterogeneity and differences in sample type and quality, the amplification of a single marker relative to other markers in the assay may vary greatly. Thus, a comparison of allele peak areas between markers, as an indicator of chromosome copy number, is not recommended. Furthermore, as only peak areas within a single locus are compared, allele dosage ratios are more resilient to the effects of the plateau phase of the PCR than other dosage assays (9).

The procedure described here utilizes a “one-tube test,” in which 12 markers are co-amplified in one multiplex reaction (*see Table 1* and *Fig. 1*). Four markers are used for both chromosomes 13 and 18, and three for chromosome 21. Primers for the amelogenin (AMEL) locus are also included for sexing purposes. Extra markers are listed, and may be used if the multiplex proves to be uninformative for any given chromosome (*see Table 1*). The chromosome 21 markers are located in the Down’s critical region (21q22.1–21q22.2), with the exception of D21S11, which is proximal to this. Chromosome 13 and 18 markers are located along the length of each chromosome to increase the chance of detecting unbalanced chromosome rearrangements. The AMEL primer set (10) exploits a 6-bp deletion on the X chromosome, and because of the close sizes of the X and Y alleles enables XXY (Klinefelter syndrome) and XYY complements to be detected.

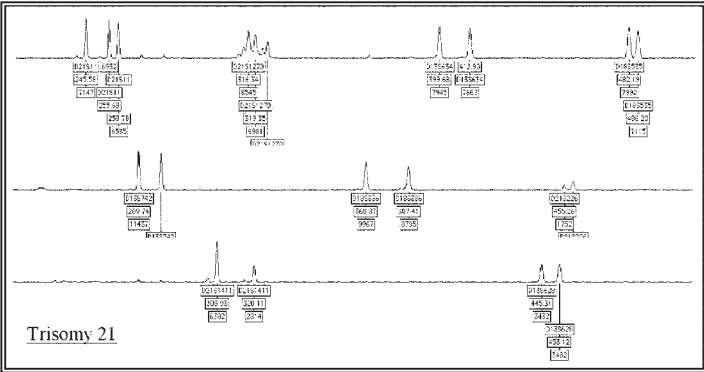
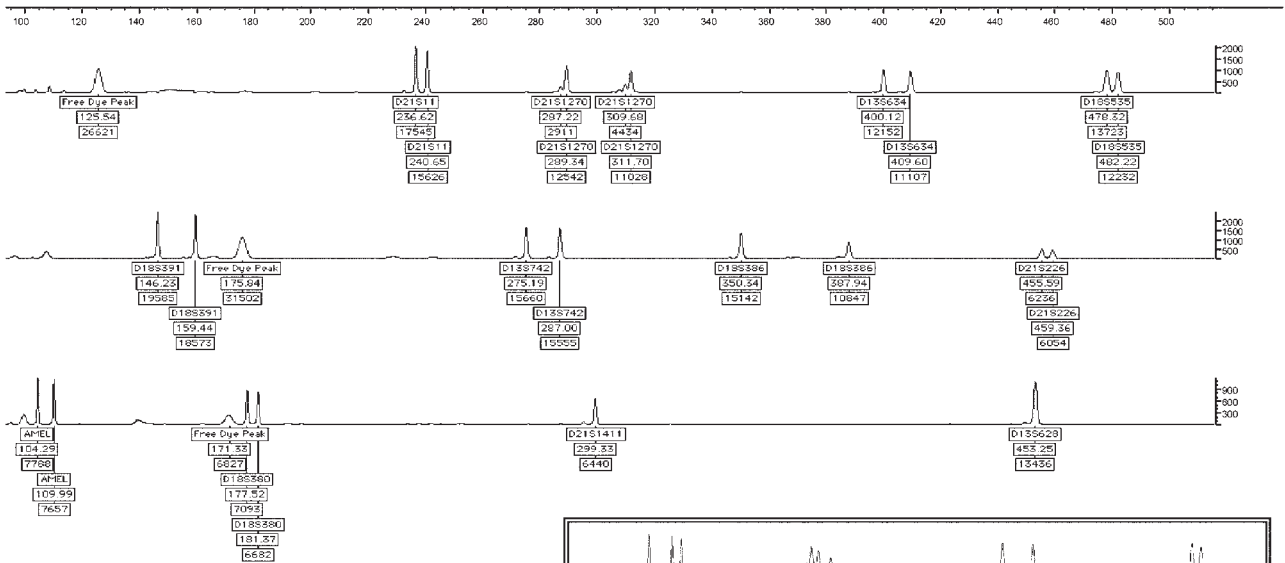
### 3. Potential Problems

#### 3.1. Maternal Cell Contamination (MCC)

Evidence of a second genotype, as shown by inconsistent dosage ratios for each chromosome and/or extra allele peaks, usually indicates contamination of

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Fig. 1. (*opposite*) Genotyper profile of a normal diploid sample amplified in the QF-PCR multiplex and analyzed on a 310 genetic analyzer. Heterozygous markers exhibit two allele peaks with peak areas in a 1:1 ratio. Size in basepairs is shown on the horizontal axis, fluorescent units on the vertical axis. Peaks are labeled with name, size, peak area. Inset is a Genotyper profile of a trisomy 21 sample. Chromosome 21 markers exhibit three alleles (D21S11 and D21S1270) or two alleles in a 2:1 ratio (D21S1411) or a 1:2 ratio (D21S226). Chromosome 13 and 18 markers exhibit normal 1:1 ratios.



the sample by maternal cells (*see Fig. 2*). This is particularly the case with bloodstained AF samples, although the degree of bloodstaining should not in itself be used as an indicator of maternal-cell contamination (MCC). In our sample set, approx 2% of AF samples are found to have two genotypes, although allele ratios vary considerably, proportional to the relative contribution of each cell line. To date, all samples have been accompanied by some degree of bloodstaining, although this ranges from a pale pink cell pellet and clear liquor to a deep red coloration of the whole fluid.

Because of skewed allele ratios, it is recommended that QF-PCR results from samples exhibiting MCC are not interpreted. However, if a Y allele is detected at the AMEL locus, it is possible to estimate the proportion of cells that are fetal in origin. Interphase-FISH may then be used if the analysis takes into account the fetal-to-maternal ratio; the number of analyzed cells can be increased to account for those that are maternal, or a sex chromosome probe can be co-hybridized with an autosome probe and only the male cells analyzed. If a Y allele is not detected, maternal DNA is required to determine which genotype is fetal in origin.

The detection of maternal cells in an AF sample should not discredit the karyotype analysis of cultured cells. Subsequent genotype analysis of cultured cells from samples showing MCC, normally demonstrates a single genotype, consistent with the selection and growth of fetal cells, and loss of maternal cells during the culture process. In those samples with a mixed female/male cell population evident on the direct analysis, a single male genotype is usually detected on the follow-up test. However, care must be taken with heavily bloodstained AF exhibiting a single female genotype, as the genotype may be maternal in origin. This phenomenon has been observed on two occasions in our sample set, with a different genotype identified on subsequent analysis of cultured cells. In both cases, the cultures showed marked delayed growth.

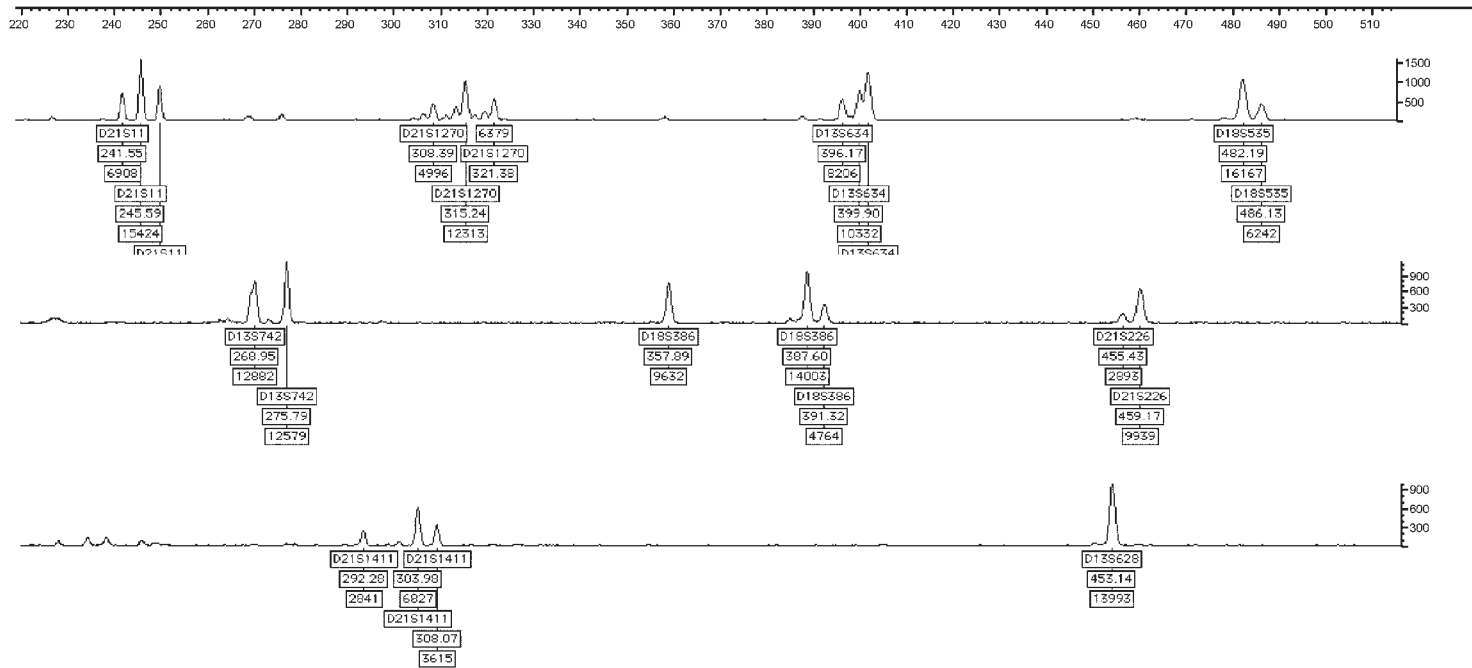
### 3.2. Mosaicism

The problem of mosaic genotypes and karyotypes in prenatal samples is well-documented, particularly in CVS. With respect to QF-PCR, two issues are relevant; the levels of mosaicism detectable by the QF-PCR technique, and the degree of concordance between a direct test result and the fetal genotype.

The first of these can only be determined by the analysis of samples (both postnatal and prenatal) exhibiting mosaicism for one of the tested regions. The

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Fig. 2. (*opposite*) A typical Genotyper profile from a bloodstained AF sample, showing maternal-cell contamination. Markers either have three alleles in unequal ratios (D21S11, D21S1270, D13S634, D18S386, D21S1411) or 2 alleles with a range of dosage ratios (D18S535, D13S742, D21S226).





**Table 1**  
**Details of Primers Used in the QF-PCR Multiplex**

Marker	Location	Heterozygosity	Size range (bp)	Primer Sequences 5'-3'	Amount $\mu$ M	Reference/Source
D13S305-F -R	13q12.1–13q14.1	0.75	430–465	<u>HEX</u> -GCCTGTTTGAGGACCTGTCGTTA TGGTTATAGAGCAGTTAAGGCAC	0.25	UTAH database
D13S628-F -R	13q31–q32	0.688	425–470	<u>NED</u> -TAACATTCATGTCCCTTACAGAT GCAAGGCTATCTAACGATAATTCA	0.6	UTAH database
D13S634-F -R	13q14.3–q22	0.812	385–440	<u>6-F</u> -GGCAGATTCAATAGGATAAATAGA GTAACCCCTCAGGTTCTCAAGTCT	0.6	<b>12</b>
D13S742-F -R	13q11–q21.1	0.75	235–315	<u>HEX</u> -ATAACTGGGCTAGGAATGGAAATA GACTTCCCAATTCAGGAGGACT	0.6	UTAH database
D18S380-F -R	18q22.3–q23	0.667	160–200	<u>NED</u> -GCATTCTGGGCAACAAAGTCAAAC GAGATAACCCAGGCAAGAACAGGA	0.2	UTAH database
D18S386-F -R	18q22.1–q22.2	0.875	330–400	<u>HEX</u> -TGAGTCAGGAGAATCACTTGGAAC CTCTCCATGAAGTAGCTAAGCAG	0.6	<b>6</b>
D18S391-F -R	18pter–18p11.22	0.75	140–180	<u>HEX</u> -GGACTTACCACAGGCAATGTGACT TAGACTTCACTATTCCTCATCTGAG	0.2	UTAH database
D18S535-F -R	18q12.2–q12.3	0.92	455–500	<u>6-F</u> -CAGCAAACCTTCATGTGACAAAAGC CAATGGTAACCTACTATTTACGTC	0.4	<b>12</b>
D21S11-F -R	21q21	0.9	225–280	<u>6-F</u> -TTTCTCAGTCTCCATAAATATGTG GATGTTGTATTAGTCAATGTTCTC	0.6	<b>13</b>
D21S1270-F -R	21q21–q22.1	0.86	285–340	<u>6-F</u> -CTATCCCCTGTATTATTCAGGGC TGAGTCTCCAGGTTGCAGGTGACA	0.8	<b>14</b>
D21S1411-F -R	21q22.3	0.933	256–340	GTAGATACATACATATGATGAATGC <u>NED</u> -TATTAATGTGTGCCTTCCAGGC	1	<b>12</b>
AMXY-F -R	Xp22.1–22.31 Yp11.2	–	X: 104 Y: 110	<u>NED</u> -CCCTGGGCTCTGTAAAGAATAGTG ATCAGAGCTTAAACTGGGAAGCTG	0.2	<b>10</b>
IFNAR-F -R	21q22.1	0.83	450–500	<u>HEX</u> -GTTCTTCATTTGATCTTAGCCATC GTGAGATAACTGGCAAGAAGATAA	0.2	<b>16</b>
D21S226-F -R	21q22.1	0.59	440–470	<u>6-F</u> -GCAAATTTGTGGATGGGATTAACAG AAGCTAAATGTCTGTAGTTATTCT	0.2	<b>15</b>

The first twelve sets of primers are multiplexed together. IFNAR and D21S226 are used as single-marker assays, as required. Size ranges given are those used in Genotyper version 2.5 (Applied Biosystems). Heterozygosity values and loci are taken from the referenced source. Many of the primer sequences have been redesigned from those published, in order to create the multiplex detailed here (UTAH database: <http://www.gdb.org>).

generation of “artificial mosaics” by the mixing of two genotypes in known measures represents a chimera rather than a mosaic genotype. Extensive analysis of the one prenatal mosaic sample identified in our sample set showed that a minimum level of 30% trisomy mosaicism could be detected. The CVS sample exhibited marked confined placental mosaicism and—because of the presence of an extra allele at three of the chromosome 18 loci—was interpreted as a meiotic non-disjunction event leading to a trisomy conceptus, followed by loss of one chromosome 18 in a placental cell line. However, a mitotic error occurring in a disomic fetus and leading to similar levels of mosaicism may be harder to detect, because of the absence of a third allele. Indeed, QF-PCR identified only one of three trisomy 18 or 21 mosaics described by Pertl et al., 1999 (6). This mosaic was also triallelic, in this case for chromosome 21.

Discrepancies between the chorionic and fetal genotype are represented, to some extent, by differences observed between analyses of direct preparations (as in the case of rapid aneuploidy tests) and cultured cells. Chorionic villi consist of an outer trophoblast layer and internal mesoderm. The mesoderm layer is derived from a later fetal-cell lineage, whereas the cytotrophoblast is derived from a much earlier lineage, and is thus less representative of fetal tissue (11). The direct DNA preparation described in **Subheading 5.2.** is likely to result in the release of DNA from cells of both the trophoblast layer and mesenchymal core. Interphase-FISH results are also believed to represent both cell layers. In contrast, karyotype analysis of direct CVS preparations concerns only the trophoblastic line, and culture conditions primarily lead to expansion of the mesoderm cell line, resulting in a final karyotype that is more representative of the fetus. The mesoderm can be exposed by digestion of the trophoblast layers with a protease, and it may be feasible to incorporate this into the QF-PCR procedure. In summary, care should be taken in the interpretation of trisomic prenatal results derived from CVS material, in the absence of an appropriate indication from ultrasonographic imaging.

## 4. Materials

Filter-sterilized water (MilliQ—Millipore, Bedford, MA) is used for all procedures.

### 4.1. DNA Preparation

1. Phosphate-buffered saline (PBS): 150 mM NaCl, 8.4 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.8 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, pH 7.4. Autoclave and store at 4°C once opened.
2. InstaGene Matrix (Bio-Rad, Hercules, CA): Store at 4°C.

### 4.2. PCR

1. T.1E buffer: 10 mM Tris-HCl pH 7.6, 0.1 mM EDTA. Filter-sterilize and store at room temperature.

2. 10X PCR buffer, magnesium-free: 670 mM Tris-HCl, 166 mM ammonium sulfate, 1.7 mg/mL bovine serum albumin (BSA), pH to 8.2 with HCl. Filter-sterilize and store at  $-20^{\circ}\text{C}$  in 500  $\mu\text{L}$  aliquots. Add 3.5  $\mu\text{L}$  14.3 M  $\beta$ -mercaptoethanol to each tube before use.
3. Deoxynucleotide triphosphates (dNTPs): These are supplied separately at concentrations of 100 mM (Promega, Madison, WI) and should be pooled and diluted to 5 mM in T.1E buffer. Long-term storage is at  $-20^{\circ}\text{C}$  in 500- $\mu\text{L}$  aliquots. To avoid repeated freeze-thawing, aliquots are stored at  $4^{\circ}\text{C}$  once in use.
4. 5' labeled fluorescent oligonucleotide primers: The author uses Applied Biosystems (ABI; Foster City, CA) HPLC-purified primers, 5'-labeled with 6-FAM, HEX, or NED. Fluorescence-labeled primers should not be exposed to light for prolonged periods of time or repeatedly freeze-thawed (*see* data sheet). Primers are split into appropriate aliquots, and are stored at  $-20^{\circ}\text{C}$ . Upon thawing, T.1E buffer is added to give a working solution of 50  $\mu\text{M}$ , and the primers are stored at  $4^{\circ}\text{C}$ .
5. Unlabeled oligonucleotide primers: The author uses Invitrogen (Grand Island, NY), desalted primers. These are supplied as precipitates and are resuspended in T.1E buffer and stored at  $-20^{\circ}\text{C}$ . Although unlabeled primers are more stable than fluorescent-labeled primers, freeze-thawing should be avoided. Stock solutions are diluted to 50  $\mu\text{M}$ , and these working solutions are stored at  $4^{\circ}\text{C}$ .
6. *Taq* polymerase: The author uses Promega enzyme supplied at 5 U/ $\mu\text{L}$  and stored at  $-20^{\circ}\text{C}$ .
7. Magnesium chloride: Supplied with the *Taq* polymerase at a 25 mM concentration. This is stored at  $-20^{\circ}\text{C}$ .

### 4.3. Analysis

1. The author uses capillary-based genetic analyzers (Applied Biosystems [ABI] models 310 and 3100). All consumables—10X EDTA buffer, polymer (POP4 and POP6), capillaries (36 cm and 50 cm), 310 tubes, septa (single tube and plate)—are purchased from ABI.
2. Deionized formamide (Toxic: refer to the material safety data sheet): HI-DI formamide (ABI) is highly deionized, in order to minimize the breakdown of the fluorescent label. It is stored at  $-20^{\circ}\text{C}$  in appropriate aliquots.
3. Genescan-500 ROX size standard: The author uses ABI size standard, designed for 100–500 bp fragment analysis.
4. Loading buffer: This contains blue dextran and is supplied with the ROX-500 size standard (ABI).
5. 96-well plates: The author uses those recommended for use on the 3100 genetic analyzer (ABI, MicroAmp Optical 96-well reaction plates).
6. Software: For fragment analysis, Genescan Analysis versions 3.1. and 3.7. are used on the 310 and 3100 genetic analyzers, respectively. For allele size-calling and labeling, Genotyper version 2.5 (ABI) is used. Microsoft Excel (Microsoft, Redmond, WA) is used to calculate and tabulate allele dosage ratios.

## 5. Methods

The processing of a number of prenatal samples at one time, and the risk of sample mix-up, necessitates stringent quality control procedures (*see Note 1*). In addition, care must be taken, as with all PCR-based tests, to avoid contamination of tested material with amplified products of previous reactions and external DNA (*see Note 2*).

### 5.1. Sample Preparation

Sample and DNA preparation procedures are carried out in a class II biological containment cabinet, up to **Subheading 5.2., step 5** (*see Note 3*).

#### 5.1.1. Amniotic Fluid

Between 10 and 20 mL AF is normally received in a 20-mL Sterile Universal Container (*see Note 4*), and any bloodstaining is recorded (*see Subheading 3.1.*). The sample is centrifuged at 200g for 10 min. Fluid is carefully removed using a 20-mL syringe to leave approx 1 mL of fluid (equal to the top of the conical section of the Universal). The cell pellet is resuspended in the fluid using a plastic Pasteur pipet and approx 100  $\mu$ L (2–3 drops) is transferred to a 0.5-mL Eppendorf tube. This aliquot (approx 1/10 of the original AF sample) can be stored at 4°C until processed. The remainder can be cultured for subsequent karyotype analysis, as required.

#### 5.1.2. Chorionic Villus and Tissue Samples (*see Note 5*)

A single villus (as opposed to a frond) is dissected from a chorionic villus sample and cleaned to remove blood, mucus, and maternal tissue. A section of that villus, approx 2–3 mm in length (*see Note 4*) is transferred to a 0.5-mL Eppendorf tube with 2–3 drops of medium to prevent the villus from drying out. This may be stored at 4°C. A similar amount of cleaned tissue sample is required.

### 5.2. DNA Preparation (*see Note 6*)

This is a quick and simple procedure, and can be successfully applied to a number of sample types and different sample qualities (*see Note 7*). Because it is based on a boiling lysis protocol, aided by vigorous vortexing, the safety issues associated with phenol/chloroform extractions are also avoided. Following cell lysis, a commercial resin (InstaGene Matrix) removes trace metal contaminants that may inhibit the PCR.

Prior to the DNA extraction, place the InstaGene Matrix on a magnetic stirrer and set at a medium speed for at least 5 min.

1. Pellet the cells/villus/tissue at 12,000g for 1 min in a microcentrifuge and carefully remove the liquid, leaving enough to resuspend the pellet (approx 10–20  $\mu\text{L}$ ).
2. Vortex to resuspend the cell pellet and add 200  $\mu\text{L}$  of PBS or  $\text{H}_2\text{O}$  to wash the sample (*see Note 8*).
3. Vortex the sample, pellet the cells, and remove the wash solution as described previously. Resuspend the cells in the remaining wash solution by vortexing.
4. Add 100 or 200  $\mu\text{L}$  (*see Note 9*) of InstaGene Matrix to the cells/villus using a wide-bore pipet tip, e.g., a Gilson p1000 tip and vortex.
5. Place the samples in a heated block and incubate at 56°C for 15 min.
6. Vortex vigorously for 10 s and then incubate at 100°C for 8 min.
7. Vortex again at high speed for 10 s and pellet the InstaGene Matrix at 12,000g for 3 min in a microcentrifuge.
8. Place the samples on ice to cool.
9. The DNA preparation should be stored at –20°C (*see Note 10*).

### 5.3. PCR Setup

Batches of PCR “kits” can be prepared in advance, tested, and stored at –20°C. These are 12- $\mu\text{L}$  aliquots of a master mix that include buffer/primers/magnesium chloride and dNTPs, but exclude *Taq* polymerase and DNA. Enzyme and DNA are added immediately prior to temperature cycling, to yield a total volume of 25  $\mu\text{L}$ . The final concentrations of the reaction components are 67 mM Tris, pH 8.8, 16.6 mM  $\text{NH}_4\text{SO}_4$ , 170  $\mu\text{g}/\text{mL}$  BSA, 10 mM  $\beta$ -mercaptoethanol, 1.5 mM  $\text{MgCl}_2$ , 2.5–25 pmol of each primer (*see Table 1*), 200  $\mu\text{M}$  dNTP and 3 U *Taq* polymerase in a total volume of 25  $\mu\text{L}$  (**Note:** DNA is added according to volume rather than concentration, as concentration is not measured).

1. To a thin-walled microcentrifuge tube (0.2 mL or 0.5 mL) containing 12  $\mu\text{L}$  of the master mix, add 3  $\mu\text{L}$  of diluted *Taq* polymerase (0.6  $\mu\text{L}$  enzyme, 2.4  $\mu\text{L}$  T.1E buffer).
2. Add 10  $\mu\text{L}$  of DNA solution (*see Note 11*), taking care not to disturb the InstaGene Matrix pellet, and mix by pipetting.
3. Add one drop of mineral oil if a heated lid is not being used, and place in the PCR machine.
4. PCR cycling conditions: Initial denaturation 94°C for 3 min followed by 25 cycles of 94°C for 30 s, 58°C for 1 min, 71°C for 2 min (*see Note 12*). Final synthesis: 71°C for 5 min followed by a 2-h incubation at 60°C (*see Note 13*).

### 5.4. Analysis

#### 5.4.1. PCR Product Preparation

Post-PCR clean-up to remove excess primers and free dye molecules is not carried out because of time constraints (*see Note 14*). The author uses both 310

and 3100 ABI genetic analyzers, and conditions specific to each are described. Standard use of these analyzers is not detailed here. Other genetic analyzers capable of fragment resolution, fluorescence detection, and quantification can also be used, and include ABI gel-based analyzers, models 373 and 377, and Amersham-Pharmacia ALF and MegaBase systems, although fluorescent labels may need to be substituted depending on the filter sets of each analyzer.

1. Prepare PCR products for analysis by the addition of 3  $\mu\text{L}$  of product to 14.6  $\mu\text{L}$  of HI-DI formamide and 0.4  $\mu\text{L}$  of Genescan-500 ROX size standard, either in tubes (310 analysis) or 96-well plates (3100 analysis) (*see Note 15*).
2. Denature at 95°C for 2 min and snap-chill on ice.

#### 5.4.2. 310 Analysis

Separate PCR products through a 50-cm capillary filled with POP4 (*see Note 16*). An 8-s injection time is suitable for most samples (*see Note 17*). The running conditions are 60°C for 30 min.

#### 5.4.3. 3100 Analysis

Separate PCR products through a 36-cm capillary array filled with POP6 (*see Note 16*). A 5-s injection time is suitable for most samples (*see Note 17*). The running conditions are 60°C for 2700 s.

#### 5.4.4. Genotyper Analysis

Macros are used to label allele peaks with marker name, size, and peak area (**Fig. 1**) (*see Note 18*). The Genotyper table is transferred to an Excel spreadsheet for allele ratio analysis.

### 5.5. Result Interpretation

The criteria listed here are based on 1200 QF-PCR prenatal tests, all of which were followed by karyotype analysis of cultured cells (*see Note 19*).

1. Normal allele dosage ratios range between 0.8 and 1.4 (*see Note 20*). Trisomy is indicated by an allele ratio greater than 1.8 or less than 0.65 or by the presence of three alleles of equal areas (*see Note 21*). All of these results are described as informative.
2. Two informative markers per chromosome are required for confident interpretation. This minimizes the risk of misdiagnosis resulting from primer-site polymorphisms and/or somatic repeat instability (*see Note 22*).
3. All inconclusive results should be repeated as single-marker assays. This includes markers that have dosage ratios that fall outside the ranges given here, any mark-

ers that have extra peaks that do not appear trisomic (e.g., they have differing peak areas) and markers that exhibit unusual features, such as stutter bands that have not previously been noted (*see* **Notes 18** and **23**).

## 6. Notes

1. To prevent sample mix-up, a minimal number of tube-to-tube transfers should be employed (three transfers are required for this protocol). Each sample transfer and analysis should be checked by another laboratory member, and the use of two identifiers per tube, such as sample number and name, aids sample tracking.
2. Contamination of a PCR by external DNA is evident by the appearance of allele peaks in the negative (no DNA) PCR control; a critical part of any PCR procedure and a reaction which should be set-up last in a series of samples. Separation of the PCR set-up and post PCR analysis areas should help to prevent contamination.
3. To ensure that DNA is prepared from the correct sample, the initial sample aliquot (*see* **Subheadings 5.1.1.** and **5.1.2.**) should be prepared one sample at a time in a class II biological containment cabinet, with only one sample in the cabinet during the procedure. As the subsequent DNA extraction (*see* **Subheading 5.2.**) is carried out without further tube transfers, DNA from a number of samples can be prepared simultaneously. This DNA can also be confidently used for subsequent PCR tests.
4. It is important that the DNA extraction is not overloaded with too much starting material. This leads to a failure by the InstaGene Matrix to remove all cell lysis products, and can result in inhibition of the PCR. In particular the larger sized markers in the multiplex may fail to amplify. If inhibition is observed, a further extraction can be used to remove the contaminants—100  $\mu\text{L}$  of the DNA extract is added to 100  $\mu\text{L}$  of InstaGene Matrix and treated as per the extraction protocol (*see* **Subheading 5.2., steps 5–9**).
5. The QF-PCR procedure can be applied effectively to solid tissue samples (e.g., skin and cartilage). This is particularly useful for confirming a prenatal diagnosis, or for a poor-quality sample where chromosome analysis of cultured cells may not be possible.
6. DNA prepared using the protocols described here may also be used for other molecular prenatal tests. The protocol has benefits over the traditional phenol-chloroform-based approach in terms of labor and time savings and reduced safety risks. However, as the extracted DNA may contain residual contaminants, its suitability for use in each test should be determined.
7. Although the procedure is generally successful in extracting DNA of sufficient quality for use in the multiplex, DNA extracted from bloodstained or discolored AF fluid may contain PCR inhibitors. These can be removed by a subsequent extraction, as described in **Note 4**.
8. Deionized water lyses red blood cells and may also aid lysis of cells in villus and tissue samples. A deionized water wash is used for all CVS and tissue samples and for bloodstained/discoloured AF. However, a PBS wash is used for all clear,



yellow, or cloudy AF samples, and bloodstained AF samples with a tiny pellet (increased cell lysis in these samples may result in critical DNA loss).

9. It is beneficial to adjust the volume of InstaGene Matrix in order to balance removal of all cell lysis products with excessive dilution of the DNA. A 200- $\mu$ L vol of InstaGene Matrix is used for CVS and tissue samples and the larger AF cell pellets (those that cover the base of the 0.5-mL microcentrifuge tube). Only 100  $\mu$ L of InstaGene Matrix is required for average and small AF pellets.
10. As the DNA prepared here is relatively crude, and cell lysis products that damage DNA may remain, the DNA should be stored at  $-20^{\circ}\text{C}$ . However, the DNA does appear to be stable for at least 3 d at room temperature, which allows some flexibility, such as transfer of the sample to another laboratory.
11. In addition to the necessary inclusion of the negative (no DNA) PCR control for the reasons given in **Note 2**, the use of a DNA control trisomic for one of the chromosomes and exhibiting 2:1 or 1:2 dosage for at least one marker is recommended. Normal allele dosage is exhibited by the other two non-trisomic chromosomes. The control not only demonstrates that the amplified DNA represents allele copy number for that procedure, but it can also be used as a standard against which any spurious background bands or free-dye peaks can be compared.
12. Samples that do not generate sufficient amplified sequences for analysis, because of low initial DNA concentration (usually evident by a very small original cell pellet) can be amplified with a greater number of cycles. The repeat procedure should always be accompanied by a trisomy control to ensure the reaction is still quantitative.
13. The 2-h incubation at  $60^{\circ}\text{C}$  is necessary, as *Taq* polymerases lacking exonuclease activity add a template-independent deoxyadenosine triphosphate (dATP) to the 3' end of amplified sequences (17). Without the  $60^{\circ}\text{C}$  incubation, a single base-pair (bp) size difference in the amplified sequences can resolve as a "split peak" on the profile and hinder analysis. This is a particular problem here because of the size of the fragments generated (100–500 bp). In this size range, the analysis systems efficiently resolve single bp differences, especially in smaller alleles.
14. Free-dye peaks are caused by the detachment of the fluorescent molecule from the labeled primer. These molecules are resolved as broad peaks, usually up to 180 bp in size (see **Note 16**), and as such can be distinguished from allele peaks. The breakdown of fluorescent primers can be minimized by the use of deionized formamide stored at  $-20^{\circ}\text{C}$ , and reduced exposure of labeled primers to temperatures above  $-20^{\circ}\text{C}$ . Free-dye molecules can be removed, along with unincorporated primers, by standard post-PCR clean-up protocols if required.
15. Accurate transfer of samples to the wells of a 96-well plate can be difficult. The risk of error can be minimized not only by the use of a multichannel pipet, but also by the addition of loading buffer containing dextran blue that is visible but does not interfere with the fluorescent analysis. Transparent piercable sheets are also available (AB Gene, Epsom, Surrey, UK) that can be sealed onto the plate, or rubber septa can be placed over wells that are not in use.

16. The POP6 polymer can be used on both the 310 and 3100 genetic analyzers if greater resolution is required, although this requires longer run times. Resolution of free-dye molecules (*see Note 14*) is not linear in respect to fragment size, but is influenced by both temperature and the separating matrix. A different polymer may be used to resolve free dye molecules that coincide with allele peaks.
17. Allele peak heights greater than 4000 fluorescent units are not analyzed. The CCD camera becomes saturated in this range, and peak fluorescence may be underrepresented. If a sample is overloaded, injection times can be reduced to accommodate differences in DNA concentration and the corresponding amplification. This is one of the advantages of the capillary-based genetic analyzers, for which a repeat injection does not require repeat sample preparation.
18. Tetranucleotide alleles demonstrate few visible stutter bands (*see Subheading 2.*), and only the main allele peak is labeled. However, some microsatellite markers (for instance, D21S1270 used in this system) contain a mix of both tetranucleotide and dinucleotide repeats and generate significant stutter bands. As D21S1270 alleles have a 40-bp size-range, the larger alleles generally exhibit more significant stutter effects than smaller alleles, presumably because of the longer repeat. It is therefore necessary to recognize and label at least the first stutter peak and include it in the allele peak area measurement.
19. As the published data describing the use of QF-PCR as a diagnostic test is limited (*5–8*), it is important to validate the QF-PCR strategy in the laboratory in which it is to be used. Control samples are required, and a pilot study is recommended prior to the implementation of a QF-PCR based aneuploidy diagnostic service, especially if primer sets are used that are not described in the published literature. It is also important to statistically validate the QF-PCR technique if it is to be used diagnostically, particularly if it is to be used as a stand-alone test. A statistical analysis of our data set that includes 1200 prenatal samples without a misdiagnosis compared to karyotype analysis, generated the following confidence intervals; less than 0.3% error rate with 95% confidence. Meta-analyses of published data may strengthen these confidence levels, although the use of different primer sets and protocols, as well as reported misdiagnoses (*6*), may complicate this approach.
20. The large normal range is necessary because of the use of tetranucleotide repeats. These can result in widely spaced alleles (up to 50 bp apart), and marked preferential amplification of the smaller allele, which in turn results in skewed allele dosage ratios. However, closely spaced alleles should exhibit less allele-specific preferential amplification, and would be expected to have dosage ratios closer to 1.0.
21. For samples that were found to have a chromosome aneuploidy, it may be appropriate to confirm the result and/or sample identification. This can be done by a repeat QF-PCR test or by another technique such as interphase-FISH or karyotype analysis of uncultured or cultured cells. The second analysis requires a second aliquot from the original sample to confirm sample identification.

22. Theoretically, polymorphisms in the primer-binding site could result in allele drop-out. This could result in a misdiagnosis if the result is used in isolation. Microsatellite repeat size somatic mosaicism has been detected in our sample set and could also result in misdiagnosis if a single marker was used independently.
23. Any results that remain inconclusive following the single-marker assay may be investigated using interphase-FISH or full karyotype analysis. The analysis of parental DNA can also be beneficial, particularly for the investigation of primer-site polymorphisms and repeat instability.

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## Fragile X Disease

Valérie Biancalana and James Macpherson

### 1. Introduction

#### 1.1. Fragile X Repeat Size Range

The term “Fragile X” can be used for either of two conditions resulting from trinucleotide repeat expansions at neighboring loci, *FRAXA* or *FRAXE*, in the Xq27–28 region; however, *FRAXA* is by far the more common condition, and only mutations at this locus cause the classical phenotype of “Fragile X syndrome,” those at *FRAXE* are responsible for a variable, milder phenotype of non-specific mental impairment. For this reason, many laboratories consider it cost-effective to test only for *FRAXA* mutations. However, a brief description of PCR techniques for *FRAXE* testing are also given (*see Note 1*).

In the vast majority of cases, *The Fragile X syndrome* is caused by expansions at a (CGG)<sub>n</sub> repeat sequence in the 5′ untranslated region (UTR) of the *FMR1* gene, which encodes an RNA-binding protein (**1,2**). Deletions and point mutations that affect the *FMR1* gene will result in the same phenotype, in most cases (but with no cytogenetically detectable fragile site). Such mutations appear much more rarely than the expansions, but this may reflect the fact that they are rarely tested for.

The normal form of the *FRAXA* CGG repeat is polymorphic and contains from 6 to about 45 repeats, usually interspersed by 1–3 AGG triplets (**2–4**). The most common alleles have 29 or 30 repeats, and more than 98% of all normal alleles are less than 45 repeats.

In *FRAXA* families, two basic types of abnormal unstable alleles may be observed:

1. “Premutations” are alleles that do not cause mental retardation and are characterized by an expansion to approx 55–200 CGG repeats, without abnormal

methylation. Premutations are unstable at meiosis, and when transmitted by a female, have a high risk of expanding to a disease-causing full mutation. This risk of transition is highly dependent on the size of the maternal premutation, and is greater than 95% for alleles with more than 100 repeats (5,6). The smallest reported allele to undergo transition to a full mutation in a single generation, as determined by compilation of results from a large number of laboratories, is 59 CGG repeats, without AGG interruptions (7). The boundary between “normal” (stable) and “premutation” alleles constitutes an intermediate range, or “gray zone” (between 45 and 55 repeats), where alleles can show some meiotic instability, but with minimal or no risk of transition to full mutation in a single generation.

2. “Full mutations” are alleles that cause mental retardation in males and 50–60% of females, and consist of over 200 CGG repeats. They are associated with abnormal DNA methylation of the neighboring CpG island, which extinguishes expression of the *FMR1* gene (8). Full mutations show both germinal and somatic instability.

Four forms of the mutation that are found in the population can be technically challenging for diagnostic testing:

- a. Small alleles that are close to the threshold of 55–65 repeats require special attention if they are found in individuals who do not have known relatives affected with the fragile X syndrome. These require accurate analysis of the CGG repeat number, using sequenced controls in the same range, to evaluate the risk that they may lead to a full mutation. If no transition to full mutation is documented in the extended family, one could in principle test for AGG interspersions, which stabilize such alleles. However, this is very rarely done in the context of a diagnostic laboratory (*see Note 2*).
- b. “Mosaic” individuals (MoMP) for a mixture of premutation and full mutation alleles (8,9) appear in general to have a risk of mental retardation similar to that of carriers of the full mutation only, although some cases present as “high-functioning” fragile X males.
- c. In rare cases, the mosaic pattern in males includes an apparently normal size fragment (MoMN), which can lead to false interpretation in PCR tests where only this fragment may be amplified.
- d. “Methylation mosaics” (MoMe) are rare individuals who show, in leukocytes, a partly unmethylated expansion in the size range of a full mutation. In most of these cases, they show a fairly homogeneous band in the 200–300 repeat range. In some cases, mental impairment appears to be less severe than in individuals with full methylated mutation. An absence of mental retardation was even reported in exceptional cases with little or no methylation (10).

## 1.2. Testing Strategy

At present, diagnostic procedures are based on Southern blotting and on PCR amplification. Tests for the presence or absence of the FMRP protein have been developed (but are not presented in this chapter; *see II*), and are of special value for patients with suggestive clinical phenotype, but no expansion, and who are suspected of having a point mutation in the *FMR1* gene.

### 1.2.1. Southern Blot (see **Fig. 1B**)

Southern blot analysis using a single enzyme (*EcoRI* with StB12.3 or equivalent probes) does not determine the methylation status, but allows the detection of full mutations and large premutations, and is sufficient for testing of mentally retarded probands. Combined detection of expansion and methylation status (with *EcoRI* and the methylation-sensitive enzyme *EagI*) is widely used. It offers a very good compromise in family studies to detect full mutations, mosaic patterns, and premutations (see **Note 3; 10,12–14**). Accurate sizing of premutations—important in females for assessing the risk of having affected children and for distinguishing large normal alleles from premutations—can be achieved by PCR.

### 1.2.2. PCR Amplification of Trinucleotide Repeats

PCR amplification of the CGG repeat in the FMR1 gene can be used as a primary screening method for Fragile X referrals, since normal males will have one allele visible in the size range of 6–55 repeats, and approx 70% of normal females will be heterozygous for two distinct alleles within this range, provided that the technique used allows discrimination between alleles that differ by a single repeat (see **Note 2**). All such cases may generally be reported on without further analysis (see **Notes 4 and 5** for exceptions). CGG repeats are notoriously refractory to PCR because of a combination of high strand dissociation temperatures and strong secondary structure. The detection of expansions is often limited to approx 100 CGGs. In cases in which no normal allele is visible in males or only one normal allele distinguishable in females, Southern blot analysis should be performed.

The basic polymerase chain reaction (PCR) method is adapted from Fu et al. (2), but there are various methods of visualizing the PCR products, depending upon the resolution required and the materials available. Here, radioactive PCR is described first because it gives the best resolution over the normal size range; suggested alternatives are silver staining, fluorescent PCR analysis, and ethidium bromide staining (see **Notes 6–8**).

This method requires very strict conditions to avoid contamination, which might lead to false-negative results (e.g., finding a normal-size fragment in a male carrying a full mutation). One possible problem is caused by the rare occurrence of mosaic individuals who carry a full mutation and an unexpected amplifiable normal-size fragment (MoMN, XXX, XXY, or XXYY) (see **Note 9**).

### 1.2.3. Microsatellite Analysis

Indirect diagnosis using linked polymorphic microsatellites is valuable because of its rapidity in a prenatal diagnostic context, especially when not



enough fetal material is available for Southern blot. Samples must be obtained from appropriate family members to determine phase. One-half of the at-risk pregnancies will be negative for the Fragile X haplotype, and linkage-based diagnosis will quickly alleviate parental anxiety in such cases. Furthermore, microsatellites are the recommended method to detect maternal contamination in the fetal sample (*see Note 5*).

## 2. Materials

Analytical-grade reagents should be used at all stages, unless otherwise indicated.

### 2.1. Southern Blot

1. The restriction enzymes *EagI* and *EcoRI* are needed. The author has experience with the enzyme *EagI* supplied by New England Biolabs (Ozyme, Montigny le Bretonneux, France) and *EcoRI* supplied by Q-Biogen (Appligene, Illkirch, France). Store at  $-20^{\circ}\text{C}$  (*see Note 10*).  
Alternative methylation-sensitive enzymes such as *BstZI* and *NruI* (Promega, Charbonnières, France) can be used in place of *EagI*, but may require different buffers and digestion conditions.
2. 1X digestion buffer: 10 mM Tris-HCl pH 7.3, 100 mM NaCl, 1 mM dithiothreitol (DTT), 100  $\mu\text{g}/\text{mL}$  bovine serum albumin (BSA), 10 mM  $\text{MgCl}_2$  for *EcoRI* digest (*see Note 11*). Prepare a 10X stock solution and store 1-mL aliquots at  $-20^{\circ}\text{C}$ .
3. Sodium dodecyl sulfate (SDS) (Merk, PolyLabo, Strasbourg, France) (*see Note 12*).
4. Spermidine-trihydrochloride (Sigma Aldrich, St Quentin Fallavier, France). Prepare a 100-mM solution, filtered through a 0.2- $\mu\text{m}$  filter, and store at  $-20^{\circ}\text{C}$ .
5. Agarose, type II, electrophoresis-grade.
6. A 20  $\times$  25 cm submarine gel electrophoresis gel tank suitable for running agarose gels.
7. An electrophoresis power pack.
8. TAE electrophoresis buffer 1X: 40 mM Tris base, 40 mM acetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA). Make a 20X stock solution: 484.4 g Tris base, 114.2 mL acetic acid and 37.2 g  $\text{Na}_2\text{EDTA}$ ,  $2\text{H}_2\text{O}$  to 5 L with distilled water. Store at room temperature (*see Notes 13 and 14*).
9. Concentrated loading buffer SBS 6X: A solution of 40% (w/v) sucrose, 0.25% bromophenol blue, 3% SDS in TAE 6X (*see Note 12*). Store at room temperature.
10. Low-melting-point agarose Ultra Pure (Gibco-BRL, Life Technologies, Cergy Pontoise, France). Prepare a 1% solution. Store 1-mL aliquots at room temperature.
11. Ethidium bromide aqueous solution 1% (Sigma Aldrich) (*see Note 15*).
12. StB12.3+StA22 plasmid (*see Note 16*) or StB12.3 plasmid.
13. Probe precipitation solution, prepared as follows: 200 mL 20 mM Tris: 5 mM EDTA+ 10 mL 5 M NaCl + 5 mL salmon sperm DNA 10 mg/mL. Store at  $4^{\circ}\text{C}$ .



14. Prehybridization/hybridization mix 40% formamide: on a plate with a magnetic stirrer bar, prepare a solution without formamide by mixing 200 g dextran sulfate with 1700 mL H<sub>2</sub>O. Only after this material has dissolved, add: 40 mL EDTA 0.2 M, 210 g NaCl, 8.6 g NaH<sub>2</sub>PO<sub>4</sub>, 13.4 g Na<sub>2</sub>HPO<sub>4</sub>, 3.2 g/L Ficoll 400 (Amersham Pharmacia Biotech, Orsay, France), 3.2 g/L polyvinylpyrrolidone (Amersham Pharmacia Biotech), Orsay, France), 20 mL salmon sperm DNA 10 mg/mL, and H<sub>2</sub>O up to a vol of 2 L. Autoclave and finally add 40 g SDS (*see Note 12*). Store at room temperature. Mix 500 mL of this preparation with 400 mL formamide (*see Note 17*) and 100 mL H<sub>2</sub>O to obtain 1 L of prehybridization/hybridization mix. Store at room temperature.
15. OLB for C used for multipriming reaction is made as follows: Solution O: 1.25 M Tris-HCl, pH 8.0 + 0.125 M MgCl<sub>2</sub>, autoclave. Store at 4°C. Solution A: 1 mL solution O + 18 μL 2-mercaptoethanol + 5 μL deoxyadenosine triphosphate (dATP) + 5 μL deoxythymidine triphosphate (dTTP) + 5 μL deoxyguanosine triphosphate (dGTP) (with 100 mM deoxynucleotide 5' triphosphate (dNTP) in 3 mM Tris-HCl, 0.2 mM EDTA). Store at -20°C. Solution B: 2 M HEPES (Amersham Pharmacia Biotech), titrated to pH 6.6 with NaOH. Store at 4°C. Solution C: Hexadeoxyribonucleotides = pd(N)<sub>6</sub> random hexamer (Amersham Pharmacia Biotech) evenly suspended (this does not dissolve completely) in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 at 90 optical density (OD) U/mL. Store at -20°C. Mix solution A, B, and C in the ratio of 100 μL:250 μL:150 μL and add 100 μL BSA 10 mg/mL, to make 600 μL OLB. Store at -20°C. The solution is good for several months with repeated freezing and thawing.
16. Klenow enzyme, exonuclease-free 10 U/μL (Amersham Pharmacia Biotech).
17. α[<sup>32</sup>P]deoxycytidine triphosphate (dCTP) radioisotope label 3000 Ci/mmol (Amersham Pharmacia Biotech).
18. Denaturing solution: 1.5 M NaCl, 0.5 M NaOH. Store at room temperature.
19. Neutralizing solution: 0.5 M Tris-HCl pH 7.3, 1.5 M NaCl, 1 mM EDTA. Store at room temperature.
20. 20X standard saline citrate (SSC): 3 M NaCl, 0.3 M Na-citrate. Store at room temperature.
21. Positively charged nylon membrane for DNA transfer—e.g., HybondN+ (Amersham Pharmacia Biotech).
22. Plastic bags and heat sealer.
23. Autoradiograph cassettes fitted with blue emitting intensifying (Amersham Pharmacia Biotech).
24. X-ray film: KODAK X-OMAT AR (Merk, Poly Labo) (*see Note 19*).
25. A -70°C freezer.
26. A shortwave ultraviolet (UV) transilluminator and UV protective visor and goggles.

## 2.2. Radioactive PCR Amplification and Polyacrylamide Gel Electrophoresis

1. 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl).
2. 25 mM MgCl<sub>2</sub>.

3. dNTPs at 100 mM concentration: dilute down to 2-mM separate aliquots of dGTP and equimolar mix of dATP, dCTP, and dTTP.
4. 7-deaza-dGTP (Roche-Indianapolis) (7-deaza-dGTP, Random primer labeling kits) at 10-mM concentration (store at  $-20^{\circ}\text{C}$  separately, not in combined solution with other dNTPs).
5. *Taq* polymerase (AmpliTaq Gold from Applied Biosystems [ABI], Foster City, CA) is recommended) at 5 U/ $\mu\text{L}$ .
6. DMSO (Sigma-Aldrich-Poole, Dorset, UK): store in small aliquots in the dark.
7.  $\alpha$ -[ $^{32}\text{P}$ ]dCTP, 110 TBq/mmol; Amersham Pharmacia Biotech, Little Chalfonts, Bucks, UK (see **Note 18**).
8. Sequencing-grade 5% or 6% acrylamide/bis-acrylamide (19:1) solution containing 7 M urea (Amresco-Solon, Ohio).
9. Ammonium persulfate, 10% solution: store aliquots at  $4^{\circ}\text{C}$  for up to 2 wk (Sigma-Aldrich-Poole).
10. N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich-Poole).
11. Formamide-loading buffer: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.
12. Electrophoresis gel rig and fittings, e.g., Sequi-Gen electrophoresis cells  $38 \times 50$  cm (Bio-Rad).
13. Electrophoresis power pack compatible with gel rig—e.g., PowerPac 3000 (Bio-Rad).
14. Plastic well-forming comb, 0.4 mm thick, 36 wells (more if required).
15. Plastic gel plate spacers, 0.4 mm thick.
16. Flat capillary loading tips, 0.4 mm, 0.5–200  $\mu\text{L}$  capacity.
17. Whatman No. 3 filter paper, cut to size of gel.
18. Autoradiographic film, blue sensitive,  $35 \times 43$  cm.

### 2.3. Microsatellite Analysis

1. dNTPs: These can be purchased individually at 100 mM concentration (Amersham Pharmacia Biotech).
2. 5X PCR buffer at 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM  $\text{MgCl}_2$  final: for 500  $\mu\text{L}$  solution, mix 250  $\mu\text{L}$  buffer II 10X (Applied Biosystems [ABI]), 150  $\mu\text{L}$  25 mM  $\text{MgCl}_2$  (ABI), 5  $\mu\text{L}$  each dNTPs at 100 mM (200  $\mu\text{M}$  final) and 80  $\mu\text{L}$   $\text{H}_2\text{O}$ . Store aliquots at  $-20^{\circ}\text{C}$ .
3. Fluorescent and nonlabeled oligonucleotide primers (ABI, CA or Gibco-BRL, Life Technologies). Prepare stock of nonlabeled primers at a concentration of 100  $\mu\text{M}$  and working stock of all primers (see **Table 1** and **Fig. 1A**) at a concentration of 10  $\mu\text{M}$  in millipore  $\text{H}_2\text{O}$ . Store appropriate aliquots at  $-20^{\circ}\text{C}$  (see **Note 20**).
4. *Taq* polymerase at 5 U/ $\mu\text{L}$ : the author has experience with *Taq* polymerase from Sigma Aldrich, ABI. Store at  $-20^{\circ}\text{C}$ .
5. DMSO: store in small aliquots in the dark.
6. Deionized formamide (Sigma Aldrich). Store aliquots at  $-20^{\circ}\text{C}$ .
7. GeneScan<sup>TM</sup>-500 TAMRA size standard (ABI). Store at  $4^{\circ}\text{C}$ .

**Table 1**  
**Oligonucleotide Primers Used in the Multiplex PCR Reaction**

Locus		Primer sequence 5' to 3'	Product size (bp)	Informativity
DXS998	998 spe	AGATCATT CATATAACCTCAAAGA	130–150	0.57
	998 com	<b>tacgcatcccagttgagacg</b> CAGCAATTTTCAAAGGC		
DXS548	548 spe	GTACATTAGAGTCACCTGTGGT	210–230	0.65
	548 com	<b>tacgcatcccagttgagacg</b> AGAGCTTCACTATGCAATG		
FRAXAC2	C2 spe	GACTGCTCCGGAAGTTGAATC	170–190	0.80
	C2 com	<b>tacgcatcccagttgagacg</b> CTAGGTGACAGAGTGAGATC		
DXS1215	1215 spe	GGGCAAACATTAACCTCTC	260–270	0.55
	1215 com	<b>tacgcatcccagttgagacg</b> GCCCTCTAAGTCATTACG		
Labeled Common	ComF	6-FAM- <b>tacgcatcccagttgagacg</b>		

The common primer (in bold) is the one selected by Warner et al. (17). Its sequence contains no self-complementary region and no strong similarity to known human sequences. The fluorescent primer is shown here with 6-FAM, but can be labeled using different fluorochromes (*see Note 20*).

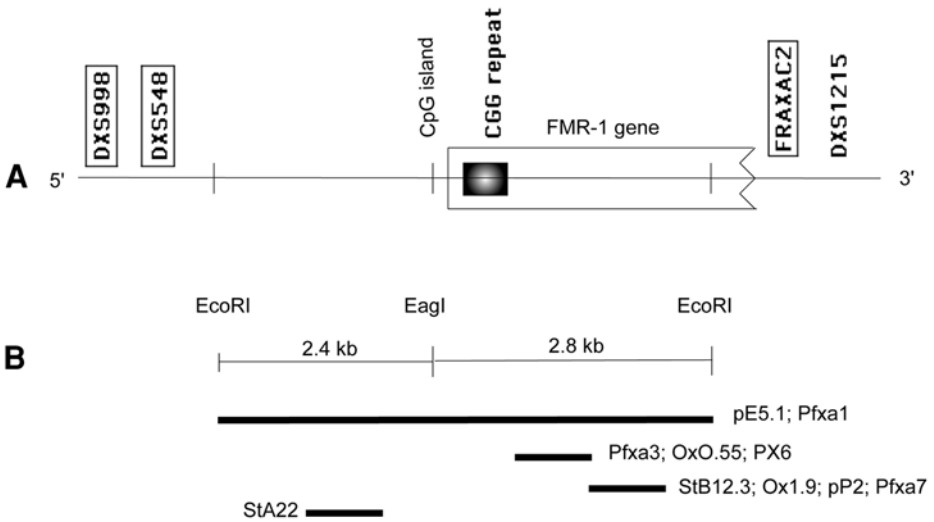


Fig. 1. (A) FRAXAC2 and DXS1215 are respectively 10 kb and 8000 kb distal of FMR1 gene, and DXS548 et DXS998 are respectively 150 kb and 400 kb proximal of FMR1 gene. A microsatellite marker, FRAXAC1, is localized 10 kb proximal to FMR1, but the high degree of linkage disequilibrium between FRAXAC1 and FRAXAC2 makes the less informative of the two (FRAXAC1) redundant for linkage analysis. (B) On the right, three major classes of probes are shown with alternate names listed. The probes StA22 and StB12.3 hybridize to a 5.2-kb *EcoRI* fragment encompassing the CGG repeat (*see Note 16*). Within this fragment lies an *EagI* site centromeric of the FMR1 gene and the CGG repeat. *EagI* is sensitive to methylation and is unable to cut the DNA if its recognition sequence is methylated. When *EagI* site is cleaved, the probe StA22 hybridizes to a nonpolymorphic 2,4-kb *EagI-EcoRI* fragment, and the probe StB12.3 hybridizes to a 2,8-kb fragment containing the CGG repetition.

8. ABI 310 DNA Sequencer and Genescan software for sizing fluorescently labeled PCR products.

### 3. Methods

#### 3.1. Southern Blot

##### 3.1.1. Restriction Digestion and Gel Electrophoresis

1. The genomic DNA must be cut with restriction enzymes prior to electrophoresis. The restriction enzyme(s) used depends on the indication of the study (*see Subheading 1.2.1.* and *Note 3*). For *EcoRI* digests: 10  $\mu$ g of genomic DNA is digested for at least 12 h in a water bath at 37°C with 30 U of *EcoRI* restriction enzyme (10 U for 4 h, and 10 U for 4 h and then 10 U again for 4 h or overnight), in a final vol of 300  $\mu$ L containing 30  $\mu$ L of 10X digestion buffer and sterile H<sub>2</sub>O. For

- EagI* + *EcoRI* digests: 3  $\mu\text{L}$  1 M  $\text{MgCl}_2$  is added to the reaction (*see Note 11*), with *EagI* enzyme (10 U for 4 h, and 10 U for 4 h and then 10 U again for 4 h or overnight).
2. Prepare a master mix of all components, except for the DNA. Distribute an adequate volume for all the samples to be analyzed in 1.6-mL microcentrifuge tubes, and then add DNA.
  3. After 1 h of digestion, add 6  $\mu\text{L}$  100 mM spermidine-trihydrochloride (*see Note 21*).
  4. After 4 h digestion, the samples should be monitored. Take 8  $\mu\text{L}$  of each restriction digest, mix with 2  $\mu\text{L}$  of SDS 6X loading buffer, and run for 1 h at 120 mA on a 1% agarose monitor gel in 1X TAE buffer (*see Note 22*). The minigel should be viewed on a UV transilluminator; undigested or partially digested samples have a characteristic streaky appearance. Dilute any uncut samples in 400  $\mu\text{L}$  final volume and add 1  $\mu\text{L}$  10X digestion mix and 10 U more of restriction enzyme(s) for 4 h or overnight.
  5. After an additional 4 h digestion, add 10 U enzyme(s) to the digests for 4 h or overnight digestion.
  6. Once the samples are completely digested, they can be precipitated after addition of 0.1% (final) SDS (*see Note 12*), 250 mM NaCl (final) and 2 vol absolute ethanol cooled at  $-20^\circ\text{C}$  (vortex after each component). Samples could be stored at  $-20^\circ\text{C}$  at this stage. Centrifuge at 13,000g for 20 min, and discard the supernatant. The DNA pellet is then washed once (without resuspension) with 300  $\mu\text{L}$  70% ethanol and centrifuged at 13,000g for 10 min. Discard the supernatant. Turn the microcentrifuge tubes upside down on tissue paper for 15 min to drain the remaining ethanol, and leave the tube to dry off under a lamp (*see Note 23*). Resuspend in 15  $\mu\text{L}$  TE 20:1 and vortex several times. Precipitated digests can be preserved 1–2 d at  $4^\circ\text{C}$  before loading.
  7. For a 20 cm wide  $\times$  24 cm long format, dissolve 1.8 g agarose in a 500-mL beaker in 230 mL TAE 1X buffer (0.8% gel; *see Note 24*). Heat the liquid (either in a microwave oven or on a hotplate with a magnetic stirrer bar) on high power until bubbles appear in the solution. Hold at boiling point for 30 s. Cool the solution to  $50\text{--}60^\circ\text{C}$  prior to casting. **Caution:** Follow local health and safety procedures for handling hot agarose.
  8. Place a comb with 6 mm  $\times$  1 mm slots in the gel former, and seal the open ends with plastic tape. When the gel has cooled down, carefully pour the liquid into the former. Check for air bubbles, especially around the comb. These can be removed using a Gilson pipet tip. Allow the gel to set for at least 1 h. When set, remove the tape and immerse the gel in 1X TAE buffer (*see Note 14*) to a depth over the gel surface of approx 2–3 mm (*see Note 25*). Remove the comb only after the gel is fully submerged in the running buffer.
  9. Add 2  $\mu\text{L}$  of SDS 6X loading buffer in all the digestion tubes, followed by 4  $\mu\text{L}$  of 1% low-melting-point agarose (heated at  $60^\circ\text{C}$ ) in each tube just before loading (*see Notes 26 and 27*). Run the gel for an initial period of 1 h at 0.5 V/cm followed by overnight electrophoresis (approx 14 h) at 2 V/cm until the bromophenol blue is at 1–2 cm from the edge of the gel.

### 3.1.2. Southern Blotting

1. After 5 min staining in an ethidium bromide solution (30  $\mu$ L of 1% ethidium bromide in 500 mL TAE 1X), photograph the gel on a UV transilluminator (*see Notes 15 and 28*) and cut off the wells and any waste agarose. Remember to slice off a corner of the gel to allow it to be orientated, and note the dimensions of the trimmed gel. Transfer the gel into a tray to be placed on a platform shaker at slow speed.
2. The gel is treated for 10 min with 0.25 M HCl (*see Note 29*), then 30 min in denaturing solution: 1.5 M NaCl, 0.5 M NaOH, followed by at least 30 min neutralization in 0.5 M Tris-HCl pH 7.3, 1.5 M NaCl, 1 mM EDTA.
3. While carrying out **step 2**, cut a piece of charged Nylon membrane (*see Note 30*), and two pieces of Whatman 3MM filter paper, to a slightly larger size than the gel. Cut one corner of the membrane to correspond to the cut corner of the gel, and use a permanent black pen to mark the membrane with an identifying number (*see Note 31*). Into a tray containing 10X SSC transfer solution, place a filter paper onto an inverted gel casting tray (this filter must be long enough to dip in the 10X SSC as a wick).
4. Add the first filter paper and the gel with the underside of the gel facing up. Squeeze any bubbles between the filter and the gel by rolling a pipet over the surface of the gel. Place the nylon membrane onto the gel and avoid introducing bubbles, with the identifying number facing the gel. Complete the blot by adding the second filter paper and a stack of paper towels to a depth of approx 6 cm. Lay a glass plate on top of the structure and compress with a suitable weight (approx 500 g). Leave to blot at least 5 h (paper towels must be changed every 15 min for the first hour and subsequently every hour) or overnight. After transfer, dismantle the blot and discard any damp paper towels. Place the underside of the membrane on a sheet of Whatman 3MM paper and re-cover with 0.4 M NaOH for 20 min for DNA fixation. Wash briefly by immersion in 2X SSC. Take care to prevent the filter from drying out at any stage.
5. The membrane is then sealed in a plastic bag of a size slightly longer than the membrane with 10 mL 40% formamide hybridization mix (for a 20  $\times$  20 cm blot), and can be either immediately prehybridized or stored at 4°C for an unlimited time.

### 3.1.3. Probe Labeling (*see Notes 18 and 32*) and Hybridization

1. Prehybridize the membrane for at least 1 h in an oven at 42°C.
2. Label 30–40 ng of probe fragment (*see Note 33*) by random priming for 1 h at 37°C or 5 h at room temperature with 4  $\mu$ L OLB for C, 2  $\mu$ L  $\alpha$ [<sup>32</sup>P]dCTP 3000 Ci/mmol, 10 U Klenow enzyme and H<sub>2</sub>O to a 20  $\mu$ L final volume. As single-stranded DNA is required as a template for the labeling reaction, denature the probe in water by heating for 5 min to 100°C in a water bath, and then cool on ice before adding the rest of the reaction.
3. Precipitate the probe by adding 180  $\mu$ L of probe precipitation solution and 500  $\mu$ L absolute ethanol (cooled at –20°C) to the reaction. Vortex, wait for 1 min, and spin for 1 min at 13,000g. Discard the supernatant, wash with 500  $\mu$ L 70°C

ethanol (room temperature), and spin for 1 min at 13,000g. Discard the supernatant and resuspend the pellet in 400  $\mu$ L TE20:5.

4. The probe is then denatured by the addition of 40  $\mu$ L 4 M NaOH (0.4 M final), and can be stored at  $-20^{\circ}\text{C}$  at this stage for up to 1–2 wk (*see Note 34*).
5. The denaturing effect of 0.4 M NaOH is stopped, and hybridization can occur when the probe is diluted in the 10 mL 40% formamide hybridization mix. Add the probe directly to the hybridization mix in the plastic bag, which is then sealed very near the membrane, but take care to avoid trapping air bubbles in the bag. Place in the  $42^{\circ}\text{C}$  oven for an overnight hybridization.

### 3.1.4. Filter Washing and Autoradiography

1. The lowest stringency washing compatible with the probe should be used to avoid losing the signal. Prepare 1 L of a wash solution (for one or several membranes) comprising 0.5X SSC and 0.1% SDS (*see Note 12*). Prepare 300 mL in a covered tray and 300 mL in a bottle in a water bath to equilibrate the solution at  $60^{\circ}\text{C}$ .
2. With a Pasteur pipet, take the hybridization solution and place into a suitable container or keep it for another use (*see Note 35*). Remove the filter with a pair of forceps and place it on a tray.
3. Add 300 mL of wash solution at room temperature for 5 min on a platform shaker at slow speed. Discard the solution in a suitable container and repeat this step.
4. Transfer the membrane on the tray with wash solution at  $60^{\circ}\text{C}$  for 15 min. Discard the solution in the container and wash again with the 300 mL contained in the bottle.
5. Wrap the filter in Saran Wrap, and in a darkroom place with a sheet of X-ray film inside an autoradiography cassette fitted with an intensifying screen. Leave to expose at  $-70^{\circ}\text{C}$  for 1 d before developing. The film can be re-exposed if the signal is not strong enough.

### 3.1.5. Blot De-Hybridization and Storage (*see Note 36*)

1. To strip a HybondN+ membrane, place it for 30 min on a tray in an platform shaker with 0.4 M NaOH.
2. Wash with 0.1 SSC, 0.1% SDS (*see Note 12*), 0.2 M Tris-HCl for at least 30 min.
3. Seal the membrane in plastic bag with 10 mL hybridization mix if needed, or 10 mL TE20.5 + SDS 1% (*see Note 12*), for long-term storage at  $4^{\circ}\text{C}$  (several years).

## 3.2. PCR Amplification of Trinucleotide Repeats and PAGE

1. The basic methodology of PCR has been described in Chapter 1. For PCR of fragments with trinucleotide repeats, especially CG-rich ones such as those found in the FMR1 gene, certain variations are advisable, notably the addition of DMSO and the use of the nucleotide analog 7-deaza-dGTP in conjunction with the regular nucleotide (*see Note 37*).
2. The following PCR mix has been found to be effective for amplifying all normal, intermediate-, and premutation-sized alleles up to approx 75 repeats (up to

100 for males, in whom there is no problem of preferential amplification of the smaller allele (*see Note 38*).

<u>Reagent</u>	<u>Quantity/reaction</u>	<u>Final concentration</u>
10X PCR buffer	1.5 $\mu\text{L}$	1X
25 mM $\text{MgCl}_2$	1.32 $\mu\text{L}$	2.2 mM
2 mM dATP/dGTP/TTP	1.5 $\mu\text{L}$	0.2 mM
DMSO	1.5 $\mu\text{L}$	10%
10 mM 7-deaza-dGTP	0.225 $\mu\text{L}$	0.15 mM
2 mM dGTP	0.375 $\mu\text{L}$	0.05 mM
Primer c	3 pmol	0.2 $\mu\text{M}$
Primer f	3 pmol	0.2 $\mu\text{M}$
$\alpha$ -[ $^{32}\text{P}$ ]dCTP	150 kBq	10 kBq/ $\mu\text{L}$
<i>Taq</i> polymerase (5 U/ $\mu\text{L}$ )	0.2 $\mu\text{L}$	0.067 U/ $\mu\text{L}$
Genomic DNA (100 ng/ $\mu\text{L}$ )	1 $\mu\text{L}$	6.7 ng/ $\mu\text{L}$ ( <i>see Note 39</i> )
<u>dH<sub>2</sub>O</u>	<u>up to 15 <math>\mu\text{L}</math></u>	
Total vol per reaction	15 $\mu\text{L}$	

- If using regular-grade *Taq* polymerase, the 1  $\mu\text{L}$  DNA should be denatured alone at 95°C under oil for a short time (5 min) before adding the remaining 14  $\mu\text{L}$  of kitmix and starting the PCR cycles. If using AmpliTaq Gold™ (ABI) or HotStarTaq™ (Qiagen), all kit components may be added at once, and the enzyme may then be activated by a single incubation of a *full* 12 or 15 min, respectively, at 95°C before cycling.
- Primers (c) and (f) encompass the CGG repeat in *FMRI (I)*. Primer sequences are: FRX-c: 5'gct cag ctc cgt ttc ggt ttc act tcc ggt and FRX-f: 5'agc ccc gca ctt cca cca cca get cct cca. Cycling conditions: (95°C 1.5 min, 65°C 1 min, 72°C 2 min)  $\times$ 30; 72°C for 7 min. Product size is 269 bp with 17 repeats, and thus the number of repeats  $n$  in a fragment of length  $L$  using these primers =  $(L-269)/3 + 17$ .
- After cycling, the samples may be analyzed immediately or stored at -20°C. Remove 5  $\mu\text{L}$  of PCR product and add to 4  $\mu\text{L}$  of formamide-loading buffer in a separate tube, mix, and denature at 95°C before loading on the gel. Polyacrylamide gels may be either 5% or 6% acrylamide, in 19:1 ratio with N,N'-methyl bis-acrylamide and with an added 7 M urea for denaturing conditions. To the gel mixture, add 0.035% (final) ammonium persulfate (AMPS) and 0.14% (final) TEMED to initiate polymerization, then immediately pour between Sequi-Gen plates (or equivalent) separated by 0.4-mm plastic spacers. The plates must be adequately sealed at the bottom, either by waterproof tape or by casting in 30 mL of polymerized gel mix. After pouring, immediately insert the well-forming comb, and add excess gel mix to allow for shrinkage. Leave to set for at least 1 h, then connect to electrodes and pre-run at 100 W until the gel temperature reaches 50°C. Next, remove the well-forming comb and load the denatured samples alongside appropriate size marker. Appropriate precautions for radiation protection should be observed (*see Note 18*). Run the gel at 50–100 W (depending upon gel size) for 3–4 h or until xylene cyanol (green) dye has reached the bottom. Then sepa-



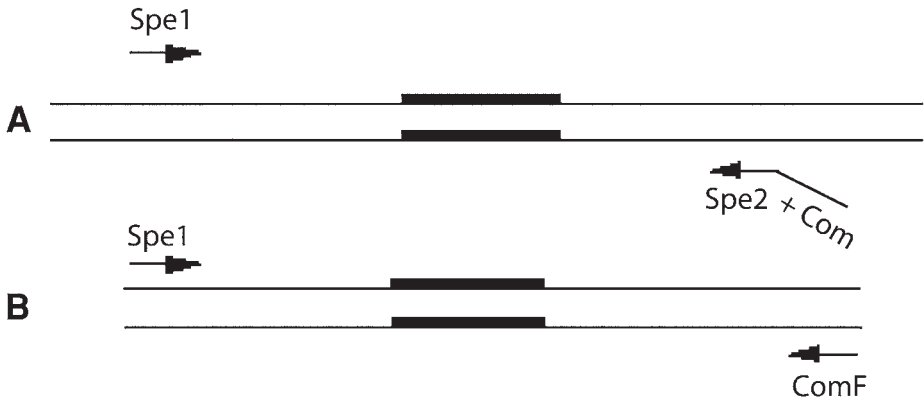


Fig. 2. Stippled box represents microsatellite repeat. Spe1 and Spe2 represent specific primers, and Com represents the tail primer. ComF is the 5' fluoresceinated primer (see **Table 1**). (A) In the early amplification cycles, specific primers give rise to a product with the tail. A 10:10:1 molar ratio of Spe1:ComF:Spe2 + Com ensures that primer Spe2 + Com is exhausted in the early amplification cycles. (B) This allows primer ComF to amplify preferentially from the ends of products from previous amplification rounds.

rate the plates with a thin wedge, remove the gel from plate onto Whatman No. 3 filter paper, enclose in polythene sheet, and overlay autoradiographic film. Allow to expose overnight at  $-70^{\circ}\text{C}$ , then develop the film and measure the band sizes (see **Note 40**).

### 3.3. Microsatellite Analysis

#### 3.3.1. Labeled Primers

For each microsatellite (see **Fig. 1A**), we use its two unlabeled specific primers and a unique fluorescent primer, in a single reaction (see **Note 20**). One of the specific flanking primers is tailed with a common 5' sequence (Com). Detection is achieved with a fluoresceinated primer corresponding to this common sequence: ComF (see **Fig. 2**).

#### 3.3.2. PCR

1. Prepare a master mix (see **Note 38**), referring to the following recipe for the volume of each reagent per sample to be amplified. PCR reactions (duplex DXS548/FRAXAC2, duplex DXS998/DXS1215 or multiplex with the four microsatellites) were performed in 25  $\mu\text{L}$  containing: 100–200 ng DNA, 20 pmole each specific primer, 2 pmole each tailed primer, and 20 pmole primer comF (see **Note 41**), 1 U of *Taq* polymerase (Sigma or Cetus), 5  $\mu\text{L}$  5X PCR buffer at 1.5 mM  $\text{MgCl}_2$  final. Keep on ice until dispensed. Add the *Taq* polymerase immediately before use.

2. To microcentrifuge tubes, add the appropriate volume of the master mix.
3. Add 1  $\mu\text{L}$  of each DNA solution (equivalent to 100–200 ng DNA), cap the tube and store on ice until ready to place in the thermal cycler.
4. Place the tubes in the thermal cycler, and perform the PCR. The samples should be denatured for 3 min at 94°C followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a 10-min final extension (see **Note 42**).

### 3.3.3. Electrophoresis and Data Analysis on ABI310

All of the features are described in the User's Manual.

1. When the reaction is complete, add 1.5  $\mu\text{L}$  of each PCR product to 16  $\mu\text{L}$  of formamide-loading buffer and 0.5  $\mu\text{L}$  of GeneScan 500 TAMRA size standard, and heat 2 min at 95°C to denature.
2. Inject for 3 s and run for 22 min.
3. The product sizes of each microsatellite are indicated in **Table 1**.

## 3.4. Interpretation of Results

### 3.4.1. Southern Blot, Hybridization Pattern of Probes StB12.3 and StB12.3 + StA22

Localization of StB12.3 and StA22 probes are shown in **Fig. 1**.

The results of digestion of DNA with *EagI* + *EcoRI* are shown on **Figs. 3A** (StB12.3 probe) and **3B** (StB12.3 + StA22 probes co-cloned in a single plasmid):

In the double digest, StA22 hybridizes to a constant 2.4 kb fragment except when the *EagI* site is methylated (this fragment serves as a useful migration control for sizing unmethylated expansions). StB12.3 hybridizes in all samples from persons with a wild-type genotype to a 2.8 kb *EagI* + *EcoRI* CGG containing fragment corresponding to the active X chromosome (lanes 5, 21). In females StB12.3 also hybridizes to an additional 5.2-kb fragment corresponding to the inactive (methylated) X chromosome (lanes 2, 18, 27, 30).

Premutations are detected as *EagI* + *EcoRI* fragments of 2.9 to approx 3.3 kb in males (lane 6) and on the active X chromosome in females, whereas the 5.3–5.7-kb *EcoRI* fragments correspond to the premutation on the inactive X chromosome (lanes 19, 25, 26). The separation between the 2.8- and 3.3-kb fragment is much better than over the 5.2-kb *EcoRI* fragment and even small premutations are easily detected (although the smallest ones may not be distinguished from the very rare alleles at the upper end of the normal range, lane 8). It should be noted that, in some female carriers of a premutation, biased X inactivation can result in an atypical two-band pattern (2.8 kb + expansion and 5.2 kb, lane 10, or 2.8 kb and 5.2 kb + expansion, lanes 1, 9). Great care should be taken to ensure that the 2.8- and 5.2-kb bands are clearly aligned with the neighboring tracks to exclude this possibility. For such cases of small

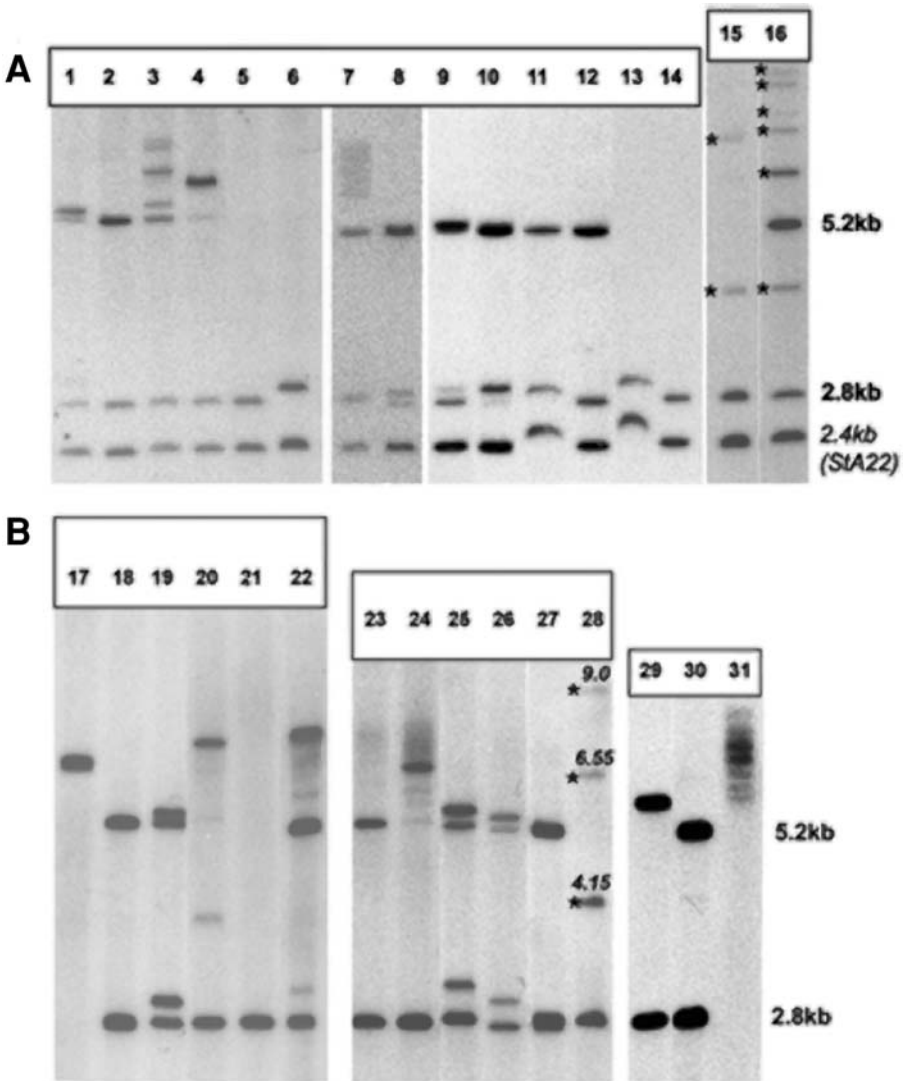


Fig. 3. Autoradiographs produced by probing Southern blots from *EagI* + *EcoRI* digests with probe StB12.3 + StA22 (panel A) or probe StB12.3 (panel B). Fragments indicated by a star (\*) correspond to an incomplete digestion of DNA, size are indicated in Panel B. See Subheading 3.4.1. for interpretation.

premutation and/or premutation with inactivation bias, the control probe (StA22) directly used together with StB12.3 is very helpful to check homogeneity of migration throughout the gel (see Note 36). Migration artefacts may occur with an inappropriate DNA extraction protocol, as shown in lanes 11

and 13 (DNA extracted from the same bloods by standard procedure are shown in lanes 12 and 14). Overloading may cause migration differences, which can be mistaken for abnormal fragments, or tight doublets may be missed if bands are too broad.

Since full mutations are methylated and not digested by *EagI*, the fragments appear as single (lanes 4, 17, 29) or heterogeneous bands (lanes 3, 7, 23, 24, 31) above 5.7 kb, detected by StB12.3 and StA22. The 2.8-kb fragment is absent in males with a fragile X mutation (lane 17), whereas full mutated females have both the normal fragments (2.8 and 5.2 kb) and mutated ones (lanes 3, 7, 23), sometimes exhibiting an inactivation bias (lanes 4, 24, 29). Note that a somatically stable full mutation (appearing as a sharp fragment) is possible in an individual, but is very unlikely to be stably transmitted in the family. In such a case, incomplete digestion in females, or restriction-site polymorphism, should be checked (we have found a family with a mutation in the *EcoRI* site distal to the CGG repeat, leading to an abnormal large fragment resembling a stable mutation; VB unpublished observation). Some full mutations may be more difficult to detect when the fully mutated fragments are very heterogeneous and the resulting smear is widely spread, especially if hybridization conditions favoring a strong signal-to-background ratio are not achieved and/or if not enough DNA is available (*see Note 28*). This is the case for some female carriers (lanes 7, 23), and for some individuals with a mosaic pattern (MoMP) or methylation mosaicism (MoMe) who are at risk of mental retardation. This would strongly affect the prediction of mental status in prenatal diagnosis. An example of a MoMP pattern with a majority of full mutated cells is shown in lane 22.

Sizes of bands generated by incomplete digestion are indicated for StB12.3 probe (a male: lane 28) and StB12.3 + StA22 probe (a male: lane 15 and a female: lane 16). Note that many stable full mutations are approx 6–7 kb, and that the rare MoMe case with bands of approx 4.0 and 6.55 kb could resemble an incomplete digest, but are identifiable because the exact pattern of an incomplete digest (in bands size and intensity) is not present (lane 20).

In the *EcoRI* digest, patterns are the same as the one observed in double digests, except that the 2.4 kb (StA22), 2.8 kb and 2.8 kb + CGG expansion bands (StB12.3) are not present.

### 3.4.2. PCR Amplification of Trinucleotide Repeats

The absence of PCR amplification should never be interpreted as establishing the presence of an expansion; confirmation by Southern blot should always be carried out. Trinucleotide repeat alleles, as with dinucleotide repeats, usually appear not as a single discrete band on a gel, but as one strong band accompanied by one or several “stutter” bands because of slippage errors during PCR,

especially when using *Taq* polymerases that lack proof-reading ability. Stutter bands are usually progressively weaker in signal at multiples of  $n$  minus the length of the repeat motif (3 bp), but stutters can also occur at  $n + 3$  bp, especially for the larger allele sizes (>35 repeats). The best estimate of allele size is thus obtained by measuring the strongest band, but it should be noted that different visualization methods (e.g., radioactive vs fluorescent) do not always agree on the exact “true” band, so there will be a standard error of  $\pm 1$  repeat for most size estimates in the normal range, and this error will be greater for large, normal, and small premutation alleles. Whenever possible, samples from the same family should be run adjacently on gels to test for allele stability. *See Figs. 4 and 5* for examples of alleles measured by different methods.

To measure the size, a calibration curve should be plotted using standard markers run on either side of the tested samples, plotting the fragment sizes against distance traveled from an origin (use the marker fragments nearest to the size of the alleles to be measured as a fixed distance from the origin, to correct for variations in migration speed across the gel). Then interpolate on the curve the distance traveled by each allelic fragment, read off its size, and calculate the number of repeats as described in **Subheading 3.2.1.**)

The size estimates obtained are generally precise enough to classify most alleles unambiguously as either normal or premutation, but those in the 45–60 repeat range require cautious interpretation (*see Notes 2 and 4*). Premutations tend to appear more as a smear on radioactive PCR gels, rather than a ladder of stutter bands. Moreover, it has been shown that almost all premutation alleles lack the interspersed AGG motifs that are characteristic of normal alleles, which is believed to explain their instability. However, techniques for measuring AGG interspersion pattern are not described here, because the precise risk estimates for such patterns have not yet been determined.

Female heterozygotes can usually be distinguished fairly easily, even if their alleles differ by only one repeat, when the stutter bands from the upper allele merge into and reinforce the lower allele (*see Figs. 4 and 5* for examples). However, if there is any doubt about the presence of two alleles in a female, Southern blotting should be carried out.

### 3.4.3. Microsatellite Analysis

The microsatellites used are described in **Table 1** and **Fig. 1A**. Very rare events of recombination between DXS548 and FRAXA or apparent gene conversion or microsatellite instability have been reported that could lead to error if indirect diagnosis was based on a single close marker.

Accurate sizing of a maternal premutation is essential for evaluating the risk of having affected offspring: the risk appears to be extremely low for an allele with less than 60 repeats, approx 50% for alleles of approx 80 repeats, and above

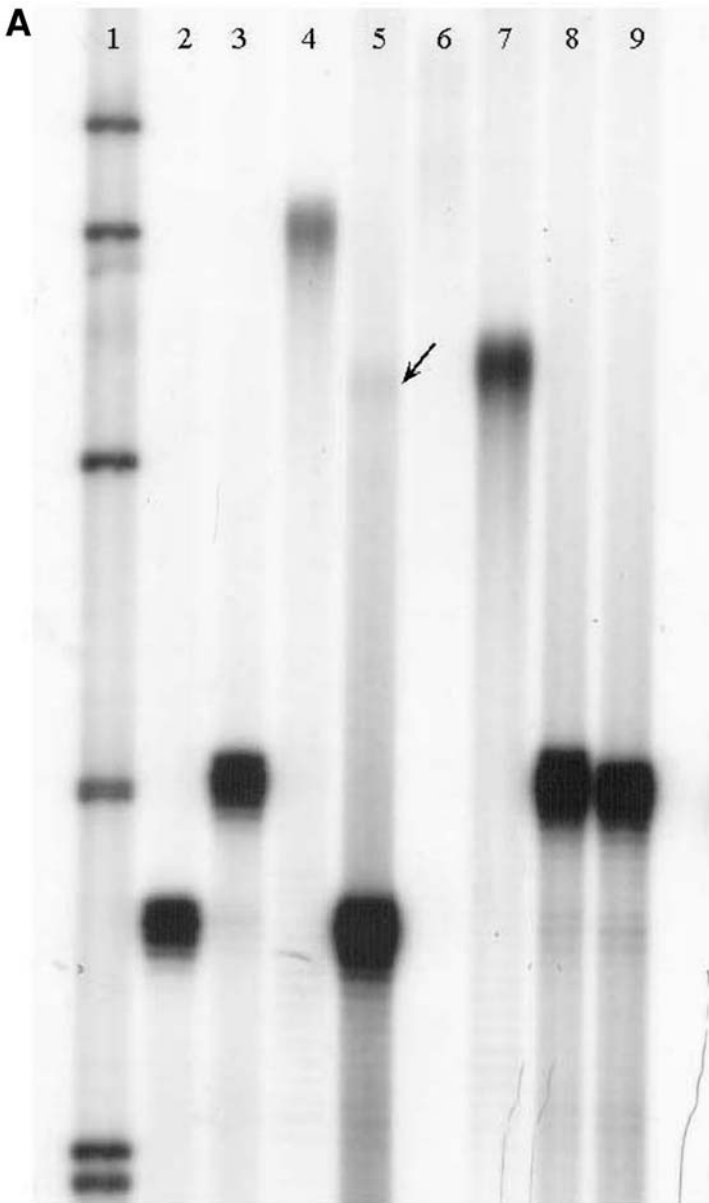


Fig. 4. (A) Autoradiograph of FRAXA radioactive PCR. Track 1: pBR322 *Msp*I size standard; 2: male, 20 repeats; 3: male, 30 repeats; 4: premutated male, approx 104 repeats; 5: premutated female, 20 + approx 73 repeats; 6: fully mutated male, no product detected; 7: premutated male, approx 78 repeats; 8: heterozygous female, 30 + 31 repeats; 9: homozygous female, 30 repeats.



Fig. 4. (B) Tracks 8 and 9 from Fig. 4A, showing the difference between a heterozygous (8) and a homozygous (9) female.

95% for alleles with more than 100 repeats (2,5–7). Very rare cases of reversion of a maternal premutation to an apparently normal allele in one offspring have been described, involving a gene conversion in at least one case (10,19–21). It is impossible to know whether these cases correspond to a true reduction in size or to undetected mosaicism for a premutation in the mother.

The fetal sample is checked for maternal contamination by comparing the fetal and maternal DNA samples (see Note 5).

#### 4. Notes

1. PCR testing for *FRAXE* follows essentially the same protocol, but the magnesium concentration may need to be slightly reduced; we have found good results using 2.0 mM. Primer sequences for the *FMR2* repeat are as follows: primer 598: 5' gcg agg aag cgg cgg cag tgg cac tgg g and primer 603: 5' cct gtg agt gtg taa gtg tgt gat gct gcc g. These primers give a fragment size of 339 bp with 17 repeats, thus repeat number  $n = (L-339)/3 + 17$ . PCR cycling conditions are the same as for *FRAXA*; the two can be combined in the same reaction if fluorescent labeling is being used (see Note 7).
2. Many laboratories use an arbitrary threshold of 45 repeats as the upper limit of the normal class, but this is by no means universally accepted. In general, for alleles over 45 repeats it is advisable to draw attention to the possibility of instability in the family when reporting the result, although the risk of an expansion to a full mutation in one generation for alleles under 60 repeats is low (7). For *FRAXE*, less data exist on unstable alleles and although known premutations again tend to be over 60 repeats, most normal alleles are below 30 repeats, which leaves a much larger zone of indeterminate stability (31–60 repeats).
3. For prenatal diagnosis, we strongly recommend the use of an *EcoRI* digest on chorionic villi because methylation of the full mutation is not always present at

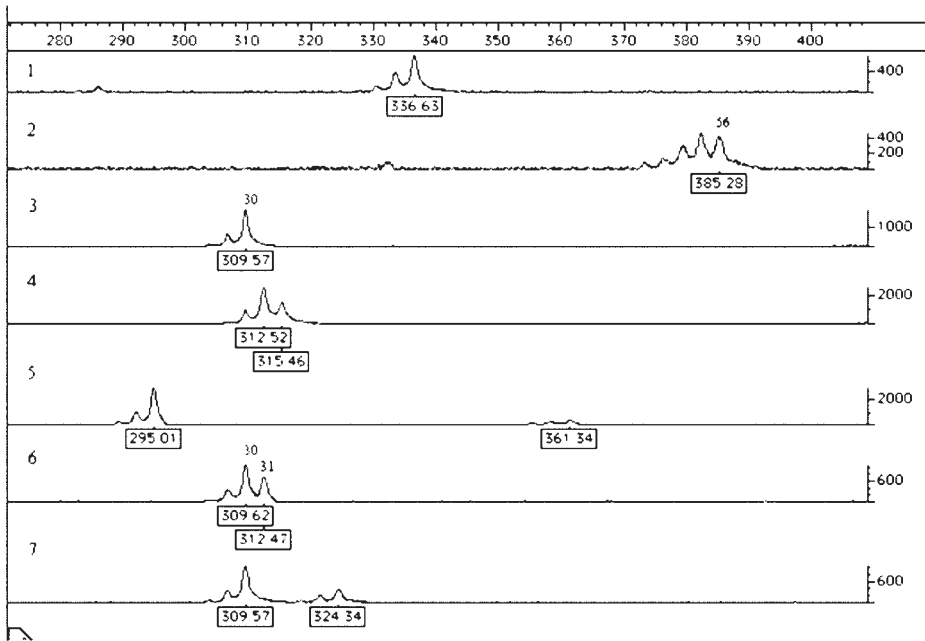


Fig. 5. Plot of FRAXA fluorescent PCR using Applied Biosystems "Genotyper™" software. Track 1: male, 39 repeats; 2: male, 56 repeats; 3: male, 30 repeats; 4: heterozygous female, 31 + 32 repeats; 5: female, 25 + 48 repeats; 6: female, 31 + 31 repeats; 7: female, 30 + 35 repeats.

the 8th–10th wk of pregnancy. This would lead to the division of a mutated signal in two classes—methylated and unmethylated fragments—which would be more difficult to detect. Nevertheless, it is very important for prenatal diagnosis to distinguish between premutations and full mutations, as they predict a completely different clinical outcome. In the case of a signal in the large premutation/small full mutation range, determination of the methylation status is critical for distinction between the two classes of mutations and an *EagI* + *EcoRI* digest must be carried out on a sample of amniotic culture or fetal blood. In the same way, the characterization of MoMe individuals requires an *EagI* + *EcoRI* digestion, in order to determine the relative percentage of unmethylated and methylated chromosomes.

4. In addition to the possibility of instability of alleles over 45 repeats (*see Note 2*), it is also recommended that a confirmatory Southern blot is carried out whenever alleles over this size are observed in case of mosaicism for a full mutation (MoMP), especially for prenatal diagnosis.
5. In prenatal diagnosis, the possibility of maternal contamination in a fetal sample must be ruled out by comparison of microsatellites in maternal and fetal samples. We have detected significant maternal contamination in 3 of 206 prenatal



- diagnoses—in one amniotic cell sample and twice in culture of chorionic villi (V. Biancalana, unpublished observations).
6. Silver staining may not give such good resolution of similar-sized alleles as radioactive PCR but is cheaper and safer, and useful if radioactive isotopes are difficult to obtain. Suitable gels for silver staining are the STURDIER™ systems (Hoefer Pharmacia Biotech Inc., San Francisco, CA). After running the gel, the plates should be carefully separated, using a wedge provided by the same supplier. The gel is then removed from the plate with minimal handling (wearing gloves) and transferred to a tray containing solution 1 (0.5% glacial acetic acid in 10% industrial-grade ethanol) for 3 min, followed by an additional 3 min in another bath of the same solution. Discard this solution and add a 1% solution of silver nitrate  $\text{AgNO}_3$  and incubate for 15 min (this may be re-used up to four times). Pour off the  $\text{AgNO}_3$  and rinse the gel in distilled water twice. Then add *freshly made* solution 3 (0.1% formaldehyde in 1.5% NaOH) and incubate for 20 min. At this point, the bands should become visible. Discard solution 3 and add 0.75% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and incubate for 10 min to fix the gel, then transfer the gel to a plastic bag and seal all around (if desired, the gel may be dried first on a vacuum drier).
  7. Fluorescent visualization is a safe and effective alternative to radioactive labeling, if the products are run on an automated sequencing apparatus such as the ABI 377. The PCR conditions are the same, except that the radioactive  $\alpha$ - $^{32}\text{P}$ dCTP is omitted and one primer is end-labeled with a fluorescent dye—e.g., FAM or HEX from ABI. PCR product is mixed with an equal volume of formamide-loading buffer and a standard size marker—e.g., TAMRA™ (labeled with a different dye)—and loaded alternately on the sequencing gel, prepared as described in Chapter 2. Data are then analyzed, and allele sizes are calculated by the automated sequencing software (e.g., Genotyper™, ABI) (15). **Note:** Size estimates for a given allele may differ slightly between fluorescent and radioactive gels. Always run control samples of a known size initially to calibrate the rate of migration of alleles.
  8. It is also possible to visualize normal *FRAXA* and *FRAXE* alleles using ethidium bromide staining of a 1.8% agarose gel, to provide a more rapid exclusion test for Fragile X (16). In this case, the proportion of 7-deaza-dGTP should be reduced to 25% of the total dGTP, and the  $\alpha$ - $^{32}\text{P}$ dCTP omitted. Load 10  $\mu\text{L}$  of PCR product with the appropriate loading buffer and standard size marker. Because the resolution of size on this type of gel is likely to be very imprecise. It is essential to include control samples of approx 50 *FRAXA* repeats for comparison (or 30 repeats for *FRAXE*). Male patients showing an allele clearly smaller than the controls may then be excluded from a diagnosis of Fragile X. However, most females and any males with a size equal to or larger than the controls, plus any patient for whom no product is observed, will need to be analyzed further by one of the other methods described in this chapter.
  9. A small population of cells with alleles in the normal range can very rarely accompany a full expansion. This would lead to a false-negative result if PCR alone was

used to exclude fragile X. In a study of 300 families, using the Fu primers (2), we have estimated that less than 1% of full mutation males have PCR-amplifiable alleles within the normal range. We have detected a carrier female with 35 CGG, 50 CGG, premutated and full mutated alleles, who might be found normal in PCR tests (V. Biancalana).

10. Authors have experience of incomplete digestion with some other combinations of enzyme suppliers and digestion buffers.
11. In the *EagI* + *EcoRI* digest, we add the two enzymes at once, but we have observed that when using the buffer conditions recommended by the manufacturers, incomplete digestion often occurred. This was relieved by the use of a higher  $\text{MgCl}_2$  concentration (20 mM), although it may be necessary to check these conditions if enzymes from different commercial sources or different buffers are used.
12. An ultra-pure SDS is necessary for the digest precipitation and for the loading buffer SBS 6X, but not for the other solutions.
13. TAE 10X is commercially available.
14. Either 1X TAE or 1X TBE buffer is suitable for use, but for high mol-wt DNA, the best buffer to decrease the tendency of the DNA to smear is TAE in combination with a low field strength (1–2V/cm). However, the buffer used and its buffering capacity influence the rate of buffer depletion. A 0.5X TBE buffer has greater buffering capacity than a 1X TAE buffer at the pH used. Smearing of DNA is evidence of buffer depletion, and this effect can be reduced by recirculating the buffer.
15. Ethidium bromide is a powerful mutagen. Gloves should be worn when handling solutions of this dye and stained gels. Also avoid skin and eye exposure to UV light.
16. The two probes StB12.3 and StA22 were co-cloned in a single plasmid, which is available upon request to Valérie Biancalana.
17. Formamide is toxic, and may cause harm to the unborn child. This chemical is also harmful when inhaled and in contact with the skin and eyes.
18. Storage and use of radioactive materials is governed by national and local safety regulations. Laboratories must ensure that appropriate permissions or licenses are obtained, that all authorized staff are adequately trained in radioactive techniques, and that local safety rules are displayed and enforced in the demarcated area. Containers that are designated for the disposal of solid or liquid radioactive wastes, specialist waste treatment company must be used.
19. Take care to avoid the use of KODAK BIOMAX MR film, which is less sensitive for this application.
20. Study of microsatellite segregation around a locus is a tool widely used. Traditionally, such a study is performed with two flanking specific primers, one of them labeled (radioactive or fluorescent) for detection. The use of radioactivity is inconvenient in a diagnostic setting, and requires an additional step of kination (end-labeling) for each microsatellite analyzed. For fluorescent analysis on automated sequencers, labeled primers are commercially available, but are rather costly and have a limited lifetime. This aspect is a real limitation when primers

are rarely used for genetic testing of very rare diseases. Here, primer sequences were chosen in order to define the best combination between one of the specific primers and the other with the Com tail. The optimal criteria for primer design are a minimum of complementarity and a  $T_m$  of approx 55–60°C (60°C is the optimal  $T_m$  of ComF). Shortening the length of the specific primer to be tailed can modulate parameters. We have validated this strategy for 55 microsatellites. Because the fluorescent primer can be labeled using different fluorochromes, several microsatellites with the same size range can thus be analyzed in the same run on a ABI sequencer. Nevertheless, if specific fluorescent labeling is used, the sequences of primers are:

LOCUS	Primer sequence 5' to 3'
DXS998:	cagcaattttcacaaggc and agatcattcatataaacctcaaaaga
DXS548	agagcttcactatgcaatggaatc and gtacattagagtcacctgtggtgc
FRAXAC2	gactgtcctcggaggtgaaactcctca and ctagggtgacagagtgagatcctgtc
DXS1215	gggcaaaacattaaacctctc and gccctctaagtcattacgct

The conditions for a PCR reaction are:

Reagent	Quantity/reaction	
	DXS548/FRAXAC2 duplex	DXS998/DXS1215 duplex
5X PCR buffer	5 $\mu$ L (1.2 mM MgCl <sub>2</sub> final)	5 $\mu$ L (1.5 mM MgCl <sub>2</sub> final)
labeled primers	25 pmole each	50 pmole DXS998 15 pmole DXS1215
nonlabeled primer	5 pmole each	5 pmole each
DMSO	2.5 $\mu$ L	
<i>Taq</i> polymerase 5 U/ $\mu$ L	0.2 $\mu$ L	0.2 $\mu$ L
Genomic DNA	100–200 ng	100–200 ng
dH <sub>2</sub> O	up to 25 $\mu$ L	up to 25 $\mu$ L
Total vol per reaction	25 $\mu$ L	25 $\mu$ L

Cycling conditions for DXS548 and FRAXAC2 duplex: (93°C for 30 s, 56°C for 1 min, 72°C for 1 min 30 s)  $\times$  30, followed by 10 min extension. Cycling conditions for DXS998 and DXS1215 duplex are : (93°C for 30 s, 50°C for 1 min, 72°C for 1 min 30 s)  $\times$  30, followed by 10 min extension (*see Note 42*). Conditions for migration are described in **Subheading 3.3.3**. Note that the sizes of PCR products are 21 bases smaller than indicated in **Table 1** because of the size of the Com tail.

- Spermidine (2 mM final) added after 1 h of the reaction improves the digestion of genomic DNA by a restriction enzyme.
- Monitor gels can be minigels made just before use or stored in a refrigerator at 4°C if wrapped in cling film.
- A vacuum-dryer should be avoided because an over-dried DNA is not easily redissolved.
- For optimal resolution during migration and transfer of DNA to the hybridization membrane, cast a gel 3–4 mm thick.
- Excessive depth will decrease DNA mobility and promote band distortion.

26. The quality of sample loading is important. We strongly advocate loading in the presence of 0.2% LMP (instead of loading buffer with 9% final Ficoll replacing sucrose), as it decreases “smiling” and gives straight regular bands. Under such conditions, most “smears” are in fact resolved in discrete bands, which are better detected and easy to differentiate from artefactual background.
27. As a partial digest may be mistaken for a mutated fragment, a control is important to accurately size the incompletely digested fragments. This could be achieved initially by including an incomplete digest, precipitated after only 1 h of digestion. An alternative is to include a plasmid-derived size marker in one lane of the gel to allow precise determination of fragment size.
28. Photography of the gel is important to check that a correct amount of undegraded DNA was loaded.
29. The depurination stage nicks the DNA and slightly improves transfer of larger fragments to the nylon membrane.
30. Hybond membrane should be handled as little as possible, always using clean, dry gloves or a clean pair of forceps.
31. The black permanent ink traps radioactivity during hybridization, and this allows the proper orientation and the identification of the autoradiograph.
32. Commercial random primer labeling kits are available—e.g., from Roche, Indianapolis, IN.
33. The probe fragment is obtained by amplification of the StB12.3 (or StB12.3 + StA22, *see* **Note 16**) plasmid insert using universal primers SK and KS. PCR reaction is performed in 100  $\mu$ L containing: 50 ng plasmid, 1 pmole primer KS, 1 pmole primer SK, 1 U of *Taq* polymerase, 20  $\mu$ L 5X PCR buffer at 1.5 mM MgCl<sub>2</sub>. Cycling conditions: (94°C for 10 s, 50°C for 10 s, and 72°C for 10 s)  $\times$  30, and 10 min extension. Check the PCR product size on an agarose minigel, running 5  $\mu$ L of the products against known size standards. If the plasmid is visible on the minigel, perform the PCR again with further dilution of the plasmid. Purify the PCR products using a Microspin 400 (Amersham Pharmacia Biotech, Orsay, France), and check the final product for concentration. Prepare aliquots with 40–50 ng and store at –20°C until needed for multiprime labeling. (Fragment StB12.3 could also be purified from a low melting point agarose gel after *Pst*I digestion of the plasmid.)
34. Denaturation with NaOH is much more convenient than heating to 100°C.
35. The hybridization mix with the probe may be kept at room temperature for another use within 10 d after 15 min denaturation at 60°C. Pour off the prehybridization solution and replace it with the retained hybridization solution.
36. The membrane can be stripped and reprobbed successfully several times (for example, with a linearity control probe such as *FMR1*cDNA, which could also detect deletion of the gene, or OXE18 probe, which detects *FRAAXE* expansion [18]).
37. Magnesium concentrations given for both the *FRAAXA* and *FRAAXE* PCR tests are higher than the standard 1.5 mM; although these levels have been found to work well in our experience, it would pay to experiment with various other concentrations until a consistently good result is obtained. The same applies to the proportion of 7-deaza-dGTP as a proportion of total dGTP.

38. In all PCR assays, great care should be taken to avoid contamination of the reaction by external sources of DNA. The most common source of contamination is by amplimers from previous reactions. The most important precaution to avoid this is always to use separate pipets for setting up the reaction and for analyzing the products. Physical separation of pre- and post-PCR areas should be instituted. The use of a laminar flow cabinet is recommended for setting up the PCR.
39. In practice, DNA concentration is not usually critical, and good results are often obtained with up to 1  $\mu$ g DNA per reaction. However, if excessive smearing or nonspecific banding is observed, quantification and dilution of the genomic DNA may be beneficial.
40. Several standard markers are available, but pBR322 digested with *MspI* (New England Biolabs, Beverly, MA) and end-labeled with  $\gamma$ -[<sup>32</sup>P]ATP gives a suitable range of fragment sizes from 622 bp down to 9 bp.
41. Fluorescent primer is light-sensitive; thus, avoid exposure to the light.
42. The PCR thermal cycling parameters have been determined with standard thickness 0.6-mL microcentrifuge tubes in a T-Gradient Thermocycler (Biometra, Biolabo, Archamps, France). The conditions may need to be adjusted if other tubes or machines are used.

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## Huntington's Disease

Linda Meredith

### 1. Introduction

#### 1.1. Background

Since the discovery of dynamic mutations approx 10 yr ago, the pathological expansion of unstable trinucleotide repeats has been shown to be the cause of an increasing number of neurological disorders. The trinucleotide repeat disorders identified to date can be categorized into two subclasses.

The first subclass, including diseases such as fragile X (CGG)<sub>n</sub> and myotonic muscular dystrophy (CTG)<sub>n</sub> has its repeat in noncoding sequences of the gene. These diseases are characterized by large (up to several thousand) repeat expansions of variable size, with somatic heterogeneity.

The second subclass is characterized by exonic (CAG)<sub>n</sub> repeats that encode a polyglutamine tract within the protein. By comparison with subgroup one, these disorders have a repeat expansion that is much smaller in size and variation. They are characterized by progressive neuronal dysfunction that typically begins in midlife and results in severe neurodegeneration (1,2).

Huntington's disease (HD) falls into the second subgroup of the so-called "polyglutamine diseases," and involves a polymorphic CAG repeat sequence located in exon 1 of the IT15 gene at 4p16.3. In individuals affected with Huntington's disease, this CAG repeat undergoes expansion from the normal range of 9–35 repeats to the pathogenic range of 40 repeats and above. Expansions of 36–39 CAG repeats define a "gray area" where the disease may not be completely penetrant (3,4).

#### 1.2. Molecular Diagnosis

Huntington's disease can be accurately confirmed or excluded in more than 95% of patients referred for diagnostic or predictive testing, using a poly-

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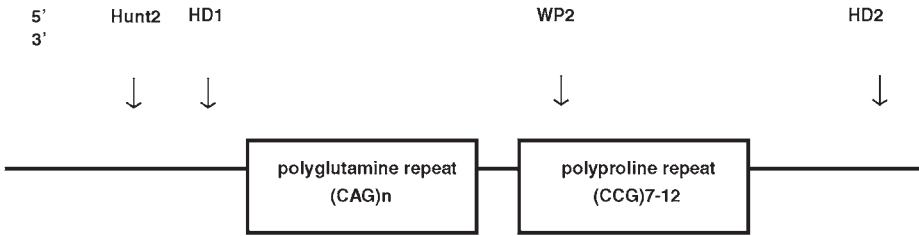


Fig. 1. Positions of CAG and CCG repeats relative to the primers used for the diagnosis of Huntington's disease.

merase chain reaction (PCR)-based assay; however, the analysis can be challenging for several reasons.

1. Adjacent and 3' to the pathogenic, polymorphic glutamine repeat, there is a second polymorphic trinucleotide repeat, coding for a series of proline residues (5,6). This polymorphic CCG repeat has allele sizes of 7–12 repeats which—if included in the amplified fragment—will result in inaccuracy in the estimation of the size of the pathogenic CAG repeat. The product obtained using the primer pair HD1/HD2, originally designed to amplify the polyglutamine expansion, includes both repeats. A second reverse primer has therefore been introduced that excludes the polyproline repeat. The primer used in the author's laboratory is WP2 (*see Fig. 1*). The product obtained using the primers HD1 and WP2 is the pathogenic repeat and diagnostic reports should be issued quoting the size of this CAG repeat alone (*see Note 1*).
2. A number of polymorphisms have been identified in the vicinity of the commonly used primers, particularly WP2. Variation of DNA sequence can affect the efficacy of primer annealing, potentially giving rise to null alleles (7–10). The result of polymorphism-induced mis-priming is amplification failure, which may result in an incorrect result on a patient. Many of the reported polymorphisms lie in the region of the WP2 primer and are indicated by a  $\uparrow$  in **Table 1**. This problem is addressed by performing additional PCR amplification.
3. There are several publications detailing various primer sets used to assay the region of the HD expansion (7–10). The methodologies described in this chapter are optimized for two analyses: The pathogenic CAG repeat only using the forward primer HD1 with the reverse primer WP2, and both CAG and CCG repeats using HD1 with the reverse primer HD2. If a single allele is obtained from the analysis of the CAG repeat only, with primers HD1/WP2, this may be the result of the amplification of one allele only with failure of amplification of the second allele, probably because of mis-priming. Further analysis must be undertaken with primers HD1/HD2 to visualize variation in the nonpathogenic CCG repeat, which often confirms the presence of two distinguishable alleles. For this reason, HD1/HD2 amplification is routinely performed alongside the HD1/WP2 amplification for all patients. All heterozygous HD1/WP2 that meet internal quality-



**Table 1**  
**Primers and Polymorphisms**

Name	Sequence 5'–3'	Product size
HD1 forward polymorphisms	atgaaggccttcgagtcctcaagtccttc <sub>↑</sub>	see below
Hunt2A forward	tggcgaccttgaaaagc	see below
HD2 reverse	gactcctcggcgactggctggcactcaa	HD1/HD2 products fall into the size range 220–592 bp
WP2 reverse polymorphisms	gtcgtcgttgctcggcggtgg ↑ ↑ ↑ ↑	HD1/WP2 products fall into the size range 71–434 bp

Primers used in the laboratory of the author. The ↑ denotes polymorphisms reported in the literature, which may cause an error in the diagnosis of Huntington's disease. HD1 and HD2 sequences are published (3). WP2 is a modification of HD3 primer (12).

control standards can be reported. For apparent homozygous HD1/WP2 results, reference can be made to the HD1/HD2 result to confirm the successful analysis of both chromosomes (for an updated analysis, see **Note 2**).

4. A homozygous result with both primer sets HD1/WP2 and HD1/HD2 is unusual. Patients who appear to be homozygous using both primer sets should undergo further testing in a specialized laboratory for analysis by Southern blotting (13).
5. The assay for the HD expansion requires PCR amplification across a very repetitive CG-rich region of DNA. The assay thus requires large amounts of both primers and the incorporation of dimethyl sulfoxide (DMSO) and "Perfect match" (Stratagene, La Jolla, CA) to improve binding specificity between the primer and the template.
6. Because of the small difference in repeat size between the normal and affected range, there is a need for accurate sizing of PCR products. Patient samples should ideally be compared against cloned universal standards in each test batch (11).
7. Because of the absolute necessity to minimize the risk of error in the presymptomatic testing of HD family members, the author's laboratory routinely tests two samples taken on separate occasions from the patient.
8. Because of the sensitive and disclosing nature of this type of testing the laboratory should document that informed consent has been obtained before testing takes place.

### 1.3. Detection of Amplified Products

1. There are various techniques that can be applied to the visualization of the CAG repeat following PCR. The method of choice in the laboratory of the author is denaturing polyacrylamide gel electrophoresis (PAGE) followed by blot hybridization using a (CTG)<sub>6</sub> oligonucleotide probe radiolabeled with <sup>32</sup>Phosphorus (see

**Note 3).** This method takes advantage of the stutter bands generated during the amplification procedure which permits accurate comparison of the ladder produced from a cloned standard sample to that of the patient sample. The method is very sensitive, and with experience, one-repeat differences can be reliably identified. It is also a specific method, as the CTG oligonucleotide will only hybridize to PCR products containing a complementary repeat, and will not hybridize to nonspecific PCR products.

## 2. Materials

Analytical-grade reagents should be used for the preparation of all reagents.

### 2.1. Polymerase Chain Reaction (PCR)

1. Expand high-fidelity PCR system (Roche Diagnostics, GmbH, Mannheim, Germany) contains *Taq* polymerase enzyme and PCR buffer. Store at  $-20^{\circ}\text{C}$  (see **Note 4**).
2.  $\text{MgCl}_2$ . Stock solution 25 mM.
3. Deoxynucleotide triphosphates (dNTPs). The four dNTPs are separately available in 100-mM stock solutions from many suppliers. They can be stored at  $-20^{\circ}\text{C}$  as a working stock of a 2-mM equimolar mix of all four dNTPs.
4. DMSO: Store at room temperature.
5. Perfect match PCR enhancer (Stratagene, La Jolla, CA). Store at  $-20^{\circ}\text{C}$ .
6. Control templates should include:
  - a. A sample with a large CAG repeat (70+) to ensure the efficient amplification of large fragments.
  - b. A sample of 35/36 repeats to define the intermediate repeat size (see **Note 5**).
  - c. A test containing no template DNA to rule out the presence of contaminating template.
  - d. A cloned sample for sizing the repeat number.

### 2.2. Restriction Enzyme Digestion

1. *Pst*I. Store at  $-20^{\circ}\text{C}$  supplied with buffer specific for *Pst*I 10X concentrated (New England Biolabs, Inc., Beverly, MA).
2. Bovine serum albumin (BSA) (Sigma) 1 mg/mL (optional).
3. Spermidine (Sigma) 100 mM (optional).
4. Control samples should include:
  - a. A sample known to have a large CAG repeat (70+) to ensure the identification of large fragments.
  - b. A sample of 35 or 36 repeats to define the intermediate repeat size.
  - c. A control sample with two normal alleles.

### 2.3. Gel Electrophoresis

#### 2.3.1. Polyacrylamide Gel Electrophoresis (PAGE) for PCR Products

1. Acrylamide gel solution, sequencing grade, Sequagel 6 (6% gel, National Diagnostics, Atlanta, GA). Care must be taken with the use of acrylamide in powdered or liquid form, as it is a neurotoxin. Read safety data sheets.

2. Sequagel Complete buffer reagent (National Diagnostics).
3. Ammonium persulfate (AMPS). Prepare a 10% solution, and store at 4°C for no longer than 7 d.
4. Electrophoresis buffer: TBE buffer made as a 10X concentrated stock solution: 0.89 M Tris-HCl, 0.89 M boric acid, 0.02 M ethylenediaminetetraacetic acid (EDTA). Adjust pH to 8.3. Store at room temperature and dilute to 1X immediately prior to use.
5. Formamide loading buffer: 95% formamide. Consult formamide safety data sheet. 0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA pH 8.0.
6. Acrylase™ (Stratagene).

### 2.3.2. Agarose Gel Electrophoresis for Southern Blot Analysis

1. Agarose Ultrapure (Sigma, St. Louis, MO).
2. TAE electrophoresis buffer made as 1 L of a 10X concentrated stock solution: 0.4 M Tris-HCl, 110 mM EDTA, 0.296 M glacial acetic acid (17 mL). Adjust pH to 8.0. Store at room temperature and dilute to 1X immediately prior to use.
3. Ethidium bromide (Sigma) stock solution 10 mg/mL. Store in a dark environment.
4. Loading buffer: 20% sucrose, 20 mM EDTA pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol

## 2.4. Blotting

1. Positively charged nylon membrane for DNA transfer—e.g., Hybond N+ (Amersham Biosciences Corp., Piscataway, NJ).
2. Denaturing solution: 1.5 M NaCl, 0.5 M NaOH. Store at room temperature.

## 2.5. Radiolabeling Primer or Probe

### 2.5.1. End Labeling Oligonucleotide

1. Synthetic (CTG)<sub>6</sub> oligonucleotide: Use 0.05-ng probe.
2. Radiolabeled nucleotide, gamma <sup>32</sup>P deoxydencosine 5' triphosphate (dATP) (Amersham Biosciences Corp.). **Caution:** Note that the use and storage of radioactive materials is governed by national and local safety regulations. Follow local rules for use.
3. Polynucleotide kinase: (T4 kinase) (Invitrogen, Groningen, the Netherlands). Supply enzyme with two buffers. Use 5X forward buffer, 10X polynucleotide kinase buffer and stop solution).

### 2.5.2. Randomprime Labeling DNA Probe

1. DNA probe: 4G6P1.7. Store at -20°C. Use 30–50 ng.
2. Random primer labeling kit. There are commercially available kits based on random hexanucleotide primers. The kit currently used in the laboratory is READY PRIME (Amersham Biosciences Corp.). Store at -20°C.
3. Radiolabeled nucleotide, alpha <sup>32</sup>P deoxycytidine triphosphate (dCTP) (3000 Ci/mmol) is commonly used. Store at -20°C. **Caution:** Note that the use and

storage of radioactive materials is governed by national and local safety regulations (Amersham Biosciences Corp.).

4. Ethanol: 100%.
5.  $\text{NH}_4\text{Ac}$ : 7.5 M.
6. TE solution: 3 M Tris-HCl, 0.3 M NaCitrate.

## 2.6. Hybridization and Autoradiography

1. Formamide loading buffer. Mix thoroughly. Store at 4°C. 9.5 mL formamide (95% v/v); 0.4 mL of 0.5 M EDTA (20 mM); 0.1 mL of 5% bromophenol blue (w/v) (0.05%); 0.1 mL of 5% xylene cyanol (w/v) (0.05%).
2. Polyethylene glycol (Sigma).
3. SSPE solution, 20X stock: 0.3 M NaCl, 0.2 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ .
4. Denhardt's solution: 100X stock. Aliquot into 20-mL sterile containers and store at -20°C. 2% Ficoll 400, 2% polyvinylpyrrolidone-360, 2% BSA.
5. Sodium dodecyl sulfate 10%.
6. Sodium pyrophosphate 5%.
7. Standard saline citrate (SSC) solution, 20X stock solution: 3 M NaCl, 0.3 M NaCitrate.
8. Prehybridization/hybridization solution: 6% PEG, 5X SSPE, 1% 100X Denhardt's, 1% SDS, 0.01% Na pyrophosphate (make up 20 mL fresh).
9. Herring sperm DNA or human placental DNA (Sigma).
10. Posthybridization wash solution: 3X SSC, and if necessary, 0.5X SSC.
11. X-ray film, Biomax MR 35 × 43 cm and 18 × 24 cm (Eastman Kodak Co., Rochester, NY).

## 2.7. Equipment

The suppliers suggested have been used by the laboratory of the author.

1. Thermal cycler (Applied Biosystems [ABI], Foster City, CA) model 480.
2. Vertical gel electrophoresis equipment (Invitrogen), Model S2 sequencing apparatus. Glass plates (39 × 33 cm and 42 × 33 cm) polypropylene spacers and shark tooth comb (0.4 mm) (*see Note 6*).
3. Electrophoresis power pack (Amersham Biosciences Corp.), Model ECPS 3000/150, capable of running at constant current/voltage/power.

### 2.7.1. Agarose Gel Electrophoresis

1. Horizontal gel electrophoresis tank to accommodate a submerged 20 × 25 cm agarose gel (Life Technologies, Paisley, UK, model: Horizon or similar).
2. A basic power pack is suitable—e.g., Life Technologies, model 400L.
3. UV Transilluminator (UVP Inc., San Gabriel, CA) and camera with red filter OR gel documentation system—e.g., Gel Doc 2000 (Biorad, Hercules, CA).
4. Oven for 80°C.
5. UV crosslinker (Amersham Biosciences Corp.).

6. A temperature-controlled oven. Hybaid U.S. (Franklin, MA) supplies a range of rotisserie ovens that accommodate cylindrical glass bottles in which a rolled membrane can be placed (*see Note 7*).
7. Radioactivity counter (Bioscan Inc., Washington, DC).
8. Hand-held geiger counter.
9. Autoradiograph cassettes fitted with blue emitting intensifying screens—e.g., high-speed X (X-Ograph, Wiltshire, UK).
10.  $-70^{\circ}\text{C}$  freezer.
11. X-ray film developer (X-Ograph).

### 3. Methods

#### 3.1. Analysis of Target DNA by PCR and PAGE

##### 3.1.1. Amplification of Target DNA by PCR

1. Internal quality control is covered in **Note 8**.
2. Prepare a master mix of all the PCR components and separately, an enzyme mix as indicated below. See reagents for working stock concentrations (*see Note 9*).
 

<ol style="list-style-type: none"> <li>a. Master mix:</li> </ol> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">Expand buffer</td> <td style="width: 20%;">2.5 <math>\mu\text{L}</math></td> <td style="width: 30%;"></td> <td style="width: 20%;"></td> </tr> <tr> <td>MgCl<sub>2</sub> (25 mM)</td> <td>1.0 <math>\mu\text{L}</math></td> <td></td> <td></td> </tr> <tr> <td>dNTP's (5 mM)</td> <td>1.0 <math>\mu\text{L}</math></td> <td></td> <td></td> </tr> <tr> <td>DMSO</td> <td>2.5 <math>\mu\text{L}</math></td> <td></td> <td></td> </tr> <tr> <td>Primers (1 ng/<math>\mu\text{L}</math>)</td> <td>1.0 <math>\mu\text{L}</math> of each</td> <td></td> <td></td> </tr> <tr> <td>Template DNA</td> <td>100.0 ng (volume equivalent, use a new tip for each sample)</td> <td></td> <td></td> </tr> <tr> <td>dH<sub>2</sub>O</td> <td>to make total of 21 <math>\mu\text{L}</math></td> <td></td> <td></td> </tr> </table>	Expand buffer	2.5 $\mu\text{L}$			MgCl <sub>2</sub> (25 mM)	1.0 $\mu\text{L}$			dNTP's (5 mM)	1.0 $\mu\text{L}$			DMSO	2.5 $\mu\text{L}$			Primers (1 ng/ $\mu\text{L}$ )	1.0 $\mu\text{L}$ of each			Template DNA	100.0 ng (volume equivalent, use a new tip for each sample)			dH <sub>2</sub> O	to make total of 21 $\mu\text{L}$			<ol style="list-style-type: none"> <li>b. Enzyme mix:</li> </ol> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%;">Expand enzyme (0.7 u)</td> <td style="width: 20%;">0.2 <math>\mu\text{L}</math></td> <td style="width: 30%;"></td> <td style="width: 20%;"></td> </tr> <tr> <td>Perfect match (0.3 u)</td> <td>0.3 <math>\mu\text{L}</math></td> <td></td> <td></td> </tr> <tr> <td>dH<sub>2</sub>O</td> <td>3.5 <math>\mu\text{L}</math></td> <td></td> <td></td> </tr> </table>	Expand enzyme (0.7 u)	0.2 $\mu\text{L}$			Perfect match (0.3 u)	0.3 $\mu\text{L}$			dH <sub>2</sub> O	3.5 $\mu\text{L}$		
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3. Add one drop of paraffin oil to each tube and place in PCR machine heating block at  $94^{\circ}\text{C}$  for 5 min.
4. With block temperature at  $90^{\circ}\text{C}$  for hot start, add 4  $\mu\text{L}$  enzyme mix. Use a clean tip for each sample.
5. Cycling conditions are as follows:  $94^{\circ}\text{C}$  for 30 s,  $64^{\circ}\text{C}$  for 45 s,  $72^{\circ}\text{C}$  for 1 min. HD1 and WP2 require 30 cycles and HD1 and HD2 require 35 cycles.
6. Final extension step of  $72^{\circ}\text{C}$  for 10 min, then store at  $4^{\circ}\text{C}$  prior to electrophoresis.

##### 3.1.2. PAGE

1. Assemble a clean set of glass plates with a spacer along each long edge of the plates and the smaller glass plate on top. Clamp the assembly together with bulldog clips and place the glass plate. "Sandwich" horizontally on a level stand in a tray to catch overflowing acrylamide.
2. Pour 60 mL of Sequagel 6 acrylamide solution into a chilled clean 250-mL beaker. Add 15 mL of complete buffer.
3. Add 600  $\mu\text{L}$  of freshly made 10% ammonium persulfate (AMPS) and mix gently by swirling. This mixture will polymerize over the following 10–30 min.

4. Draw the acrylamide up into a 50-mL disposable syringe slowly to avoid introducing bubbles into the mixture, and slowly expel the gel solution along the edge of the larger plate overlap. Continue until the gap between the plates is full, and then place the straight edge of the shark tooth comb between the plates at the overlap edge of the glass plates, pushing the flat edge of the shark tooth comb approx 3–4 mm into the acrylamide-filled space.
5. Place bulldog clips over the glass edge containing the combs to ensure a tight fit for combs when reinserted. Allow to polymerize for at least 30 min.
6. Remove the bulldog clips and fit the polymerized gel vertically into the electrophoresis assembly with the smaller glass plate at the back of the tank.
7. Place electrophoresis buffer (1X TBE) in the upper and lower buffer chamber so that the buffer is in contact with the gel, taking care to expel all bubbles along the gel edges.
8. Remove and refit the comb with the shark tooth edge inserted into the acrylamide by approx 1 mm.
9. Attach electrodes and set the power to 70 W. Allow the gel to warm to approx 50°C for 30 min prior to sample loading.

### 3.1.3. Sample Preparation and Electrophoresis

1. To each of the test samples and controls, add 12.5  $\mu$ L formamide-loading buffer. Place the samples on a heating block at 94°C for 5 min, then cool on ice to minimize re-annealing of the denatured DNA.
2. Load approx 6  $\mu$ L of the RS1/RS2 samples into the spaces between the teeth of the shark tooth comb (depending on well size and amplification efficiency), using thin gel-loading tips (*see Note 10*).
3. Switch on power set at 70 W and run the gel for 90 min.
4. The electrophoresis is paused to load the HD1/WP2 products and then resumed for an additional 90 min.

### 3.1.4. DNA Transfer

1. Disconnect the electricity supply and remove the glass/gel sandwich. Place horizontally on a level stand in a tray with the smaller glass plate on top.
2. Carefully remove the smaller plate by inserting a thin metal spatula between the plates and twisting. (The acrylase treatment should prevent the gel being removed with the smaller plate.)
3. Cut a sheet of Hybond-N membrane to cover the number of lanes containing samples, and soak in 1X TBE buffer. Place on top of gel avoiding air bubbles. Place six sheets of 3 MM paper, on top of the membrane, two soaked in 1X TBE and four dry. Place the front/top glass plate and a weight on top of assembly, and allow transfer to proceed overnight.
4. After blotting, carefully dismantle the transfer apparatus. Before separating the gel and the membrane, mark the membrane complying with internal quality control. Remove membrane, rinse briefly, and air-dry. The membrane is now ready for the DNA to be fixed.

5. Fix the DNA by oven baking at 80°C for 10 min followed by UV crosslinking. For alternative fixing methods, *see* **Note 11**.

### 3.1.5. Radiolabeling of Oligonucleotide

**Caution:** When using radioactive isotopes, always adhere to the local radiation protection rules for tip and waste disposal. See local standard operating procedures on radioisotope usage.

1. Place in a 1.5-mL Eppendorf tube in the following order, to yield a final vol of 20  $\mu\text{L}$ :

sterile H <sub>2</sub> O	10.5 $\mu\text{L}$
5X polynucleotide kinase buffer	4.0 $\mu\text{L}$
(CTG) <sub>6</sub> concentration 25 ng/ $\mu\text{L}$	2.0 $\mu\text{L}$
polynucleotide kinase ( <i>see</i> <b>Note 12</b> )	1.5 $\mu\text{L}$
$\gamma^{32}\text{P}$ dATP at 3000 Ci/mmol	2.0 $\mu\text{L}$
2. Incubate at 37°C for 1 h. The probe is now ready to be added to the hybridization mixture.

### 3.1.6. Hybridization

1. Place 10-mL of hybridization solution in a clean Hybaid hybridization bottle. Roll the membrane, pre-wetted with 2X SSC around a clean 25-mL pipet, with the DNA side inward. Remove the pipet and place the rolled filter inside the bottle.
2. Screw on cap tightly and roll the bottle around so that the membrane unrolls to fit the glass bottle snugly, and to ensure that the membrane is coated with hybridization solution.
3. When placing the tube in the rotisserie, make sure that the tube is placed so that the membrane is unwound in a counter-clockwise direction (as the rotisserie rotates clockwise). If the tube is placed in an opposite orientation, the membrane will roll back up and prevent efficient hybridization.
4. Prehybridize for 15 min at 42°C.
5. Add the labeled oligonucleotide to the solution. Be very careful to avoid placing the probe directly on the membranes, as this will cause excessive background (*see* **Note 13**). Hybridize at 42°C for a minimum of 3–4 h, but this step can be left overnight.
6. Remove the hybridization bottle from the oven and pour the hybridization solution containing the radiolabeled oligonucleotide away. **Caution:** This solution is radioactive, and disposal procedures must follow local rules for disposal of radioactive solutions.
7. Remove the membrane from the bottle into a large, watertight plastic box containing approx 1000 mL of 2X SSC wash solution. Agitate gently and discard as radioactive waste. Replace with a second 500 mL of the wash solution and place in a slowly shaking water bath for 15 min at 42°C.
8. Repeat the washes until there is no more radioactivity removed from the membrane as determined using a hand-held geiger counter. Remove the membrane from the last stringency wash, wrap in plastic food film, and expose to X-ray film at –70°C overnight.

9. Develop the exposed X-ray film. If the signal is too strong, repeat the autoradiography for a few hours only (*see Note 14*).

### 3.1.7. Alternative Methods for Visualization of PCR Products

1. For laboratories with no specialized facilities for working with radioisotopes, the PCR products can be visualized using silver staining of polyacrylamide gels (*see Note 15*).
2. Increasingly, quantitative analysis of fluorescent PCR products is being applied to the analysis of the HD gene, using the automated DNA sequencer 377 or 310 available from ABI (*see Note 16*). Product formation is quantified in the log phase of the reaction, permitting the discrimination of products produced by a single allele or by two alleles of the same size. In this case, additional analysis by Southern blot may not be necessary.

## 3.2. Analysis of Target DNA by Southern Blot Transfer of Genomic DNA

### 3.2.1. Restriction Enzyme Digestion of Genomic DNA

1. Prepare a master mix of all the digestion components except the DNA and water (*see Note 9*).
 

Per sample: Restriction enzyme buffer 10X conc	2.0 $\mu$ L
<i>Pst</i> I (10 U)	1.0 $\mu$ L

 BSA 2  $\mu$ L and spermidine 1.6  $\mu$ L may be added to DNA that is resistant to digestion. Dispense 3  $\mu$ L (6.6  $\mu$ L) into each labeled test Eppendorf tube.
2. Calculate the volume of each test sample, which is equivalent to 5  $\mu$ g of DNA. Calculate the volume of sterile H<sub>2</sub>O needed for a final volume of 20  $\mu$ L. First add the water, and last, using a clean tip for each sample, the test DNA. This minimizes the potential for sample cross-contamination (*see Note 17* and **Table 2**).
3. Spin tubes for 5 s in a microcentrifuge and incubate at 37°C for at least 3 h or overnight.
4. Check digestion by running a 2- $\mu$ L aliquot on a small 1% agarose gel (*see Note 18*).

### 3.2.2. Agarose Gel Electrophoresis

1. Place a clean, dry casting tray 20  $\times$  15 cm in size on a horizontal surface and seal the open sides with autoclave tape, ensuring a watertight contact with the tray surfaces.
2. Weigh out 1.8 g of agarose and add slowly while stirring to 120 mL 1X TAE buffer in a 1-L conical flask. **Note:** Do not allow the agarose to accumulate into lumps in the cold buffer, as these will remain in the boiled agarose. **Caution:** Heating liquids in a microwave oven can be hazardous. Ensure that you read the risk analysis and Standard Operating Procedure for this activity. Heat the gel in a microwave oven on medium high for 3–4 min until the agarose is completely dissolved. Gently swirl the solution occasionally to facilitate the dissolving process.



**Table 2**  
**Volume Containing 5 mg DNA**

$\mu\text{g/mL DNA}$	Volume $\mu\text{L}$	$\mu\text{g/mL DNA}$	Volume $\mu\text{L}$	$\mu\text{g/mL DNA}$	volume $\mu\text{L}$
1–172	29	251–263	19	501–555	9
173–178	28	264–277	18	556–625	8
179–185	27	278–294	17	626–714	7
186–192	26	295–312	16	715–833	6
193–200	25	313–333	15	834–999	5
201–208	24	334–357	14	1001–1250	4
209–217	23	358–384	13	1251–1666	3
218–227	22	385–416	12	1667–2499	2
228–238	21	417–454	11	= 2501	1
239–250	20	455–500	10		

Do not boil the solution, as this will evaporate the buffer and consequently alter the gel concentration.

- Cool the gel solution to approx  $60^{\circ}\text{C}$ , or just hand-hot. Add 5  $\mu\text{L}$  ethidium bromide and mix thoroughly. **Caution:** Ethidium bromide is a powerful mutagen/carcinogen. Handle with caution. Read risk assessments and SOPs before proceeding.
- Ensure that the gel casting tray is horizontal before pouring the molten gel into the tray. Remove any bubbles with a pipet tip. Insert a 20-cm comb with  $20 \times 1$  mm wide teeth. Allow the gel to polymerize, typically for 30–60 min.
- Remove the tape from the ends of the casting tray containing the cooled set gel and place the gel in the gel tank. Cover the gel completely using 1X TAE electrophoresis buffer.
- Remove the comb carefully to avoid damaging the sample wells. The gel is now ready for use.

### 3.2.3. Sample Preparation

- Prepare samples for loading by adding approx 4  $\mu\text{L}$  (1/10 vol) of bromophenol blue xylene cyanol loading buffer to each sample.
- Carefully load each sample and the controls into the wells of the gel, using a micropipet. Note the positions in which samples were loaded on the worksheet to allow accurate interpretation of results.
- A size marker—e.g., lambda DNA digested with *HindIII* may also be loaded to allow monitoring of the sample run.
- Place the lid on the electrophoresis apparatus and connect the power leads. DNA is positively charged, and therefore runs toward the negative electrode. Connect the electrodes so that the positive is at the “well end” of the gel.

5. Connect apparatus to the power supply and turn on the current. A typical gel is run at 120 V for 6 h, changing the TAE every 1.5 h to avoid overheating (*see Note 19*).
6. When the blue marker dye has run approx 20 cm, turn off the current and disconnect the power supply. Remove the gel tank lid, and holding the gel tray at the open ends, carefully lift the gel into an instrument tray.
7. In a darkroom, photograph the DNA under ultraviolet (UV) light on a transilluminator. **Caution:** Wear eye protection. Use either an electronic or Polaroid (film type 667) camera and a red gelatin filter or a gel documentation system.
8. Dispose of running buffer using an ethidium bromide exchange column if available, or dispose by pouring down the sink with plenty of water.

#### 3.2.4. DNA Transfer by Southern Blot

1. Remove the casting tray from beneath the gel in the instrument tray and cover the gel with 500 mL denaturing solution. Place on a shaking platform for 30 min.
2. Set up Southern blot to transfer the DNA to Hybond N+ using 0.4 M NaOH. Leave overnight.

#### 3.2.5. Radiolabeling of DNA Probe

1. Mix 2  $\mu\text{L}$  of DNA probe (10–30 ng/ $\mu\text{L}$ ) with 12  $\mu\text{L}$  sterile  $\text{H}_2\text{O}$  and denature by heating at  $>95^\circ\text{C}$  for 10 min, then chill on ice.
2. Add 5X Readyprime labeling mix      4  $\mu\text{L}$   
alpha  $^{32}\text{P}$ dCTP                              2  $\mu\text{L}$       (total of 20  $\mu\text{L}$  reaction mixture)
3. Incubate at  $37^\circ\text{C}$  for 10–30 min (1 h if in LMP agarose) (*see Note 20*).

#### 3.2.6. Hybridization and Autoradiography

1. The membrane is prehybridized by incubation in a suitable volume of hybridization solution at  $65^\circ\text{C}$  for 30 min. Ensure that all surfaces of the filter are bathed in the solution.
2. Denature 100- $\mu\text{L}$  human placental DNA by boiling for 5 min, then add to prehybridization solution.
3. Boil the radiolabeled probe, then quench on ice for 2–5 min. Add to the prehybridization solution.
4. Hybridize by incubating at  $65^\circ\text{C}$  for 6 h or overnight.
5. Remove the filters from the radioactive solution and wash in 100 mL of 3X SSC solution for 15 min to remove excess hybridization solution. The wash stringency is reduced to 0.5X SSC for 20 min. Repeat if necessary.
6. Check the background on the filter using a hand-held Geiger counter. When the counter reads under 20 cpm, the membrane should be placed on a flat surface (an old autoradiograph is useful for this) and covered with cling film. (This prevents the membrane from sticking to the unexposed autoradiograph as it dries out.)
7. In a dark room, place unexposed X-ray film in a cassette and place the cling film-covered membrane next to the film.
8. Autoradiography is conducted in a freezer at  $-70^\circ\text{C}$ . An interpretable result is usually obtained following a 2–3 d exposure (*see Note 22*).

## 4. Interpretation of Results

### 4.1. Size Ranges for the CAG Trinucleotide Repeat

normal	9–26
intermediate ( <i>see Note 23</i> )	27–35
reduced penetrance ( <i>see Note 24</i> )	36–39
affected	40–100+

### 4.2. Results Obtained by PCR and Polyacrylamide Gels

#### 4.2.1. Autoradiography of Result Obtained Using HD1/HD2 and HD1/WP2 (*see Fig. 2*)

Lanes 1 and 2) Duplicate samples of presymptomatic patient. Allele sizes of 18 and 27 repeats exclude the risk of developing HD.

Lane 3) Diagnostic patient. Allele sizes 21 and 45 repeats confirming the diagnosis of HD.

Lane 4) Control for 19 and 38 repeats.

Lane 5) Control for 17 and 90 repeats.

Lane 6) Control for 18 and 34 repeats.\*

Lane 7) Water blank.

Lane 8) Homozygote for 17-repeat allele\*\*

Lane 9) Diagnostic sample, 19 and 42 repeats confirming the diagnosis of HD.\*\*\*

#### 4.2.2. Autoradiograph of Result Obtained by Southern Blot (*see Fig. 3*)

### 4.3. Sizing of Alleles

#### 4.3.1. PCR Alleles

The allelic band is designated as the strongest band at the top of the stutter (*see Note 25*). The number of fainter stutter bands often increases with increasing repeat length, and this characteristic can be invaluable in the interpretation of HD autoradiographs, for which accurate sizing of the repeat is critical for the correct interpretation of the test. Using the control samples of known repeat

\*One allele seen only using primer pair HD1/HD2. Successful amplification has been achieved with this sample previously; therefore, in this case, preferential amplification of the larger allele has occurred.

\*\*The primers HD1/HD2 analysis failed. HD1/WP2 appears to be homozygous; therefore, the HD1/HD2 analysis must be repeated to test for two normal alleles, or refer sample for Southern analysis).

\*\*\*One allele seen only using primer pair HD1/HD2. This sample may have a primer-site mutation on one chromosome. It is of no consequence, as the HD1/WP2 analysis confirms the clinical question.

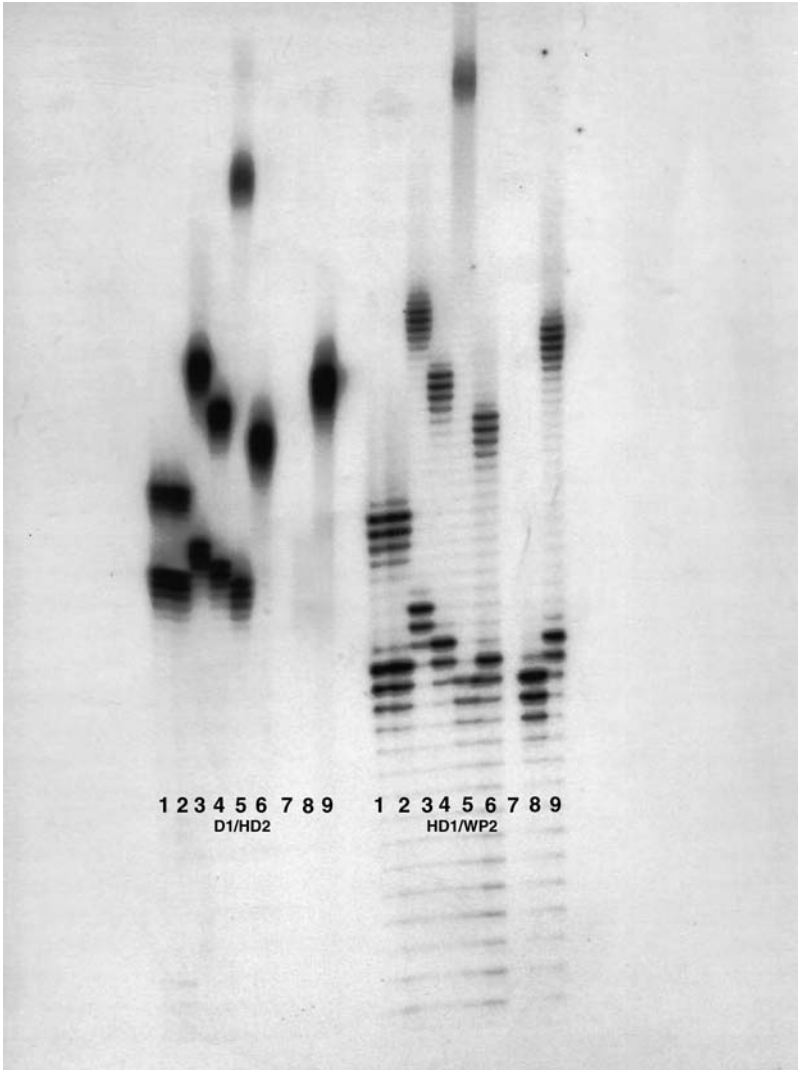


Fig. 2. Autoradiography of PCR-amplified CAG repeat using primers HD1/HD2 (left) and HD1/WP2 (right). Sizes estimated and reports written using HD1/WP2 amplification.

number included on each gel, the ladder of stutter bands can be counted and the size of the test sample can be accurately estimated. A disadvantage of the stutter bands is that the banding pattern for an individual with two alleles that differ by only one repeat can be difficult to interpret. With experience, the intensity of the allelic band can be distinguished and a reliable interpretation

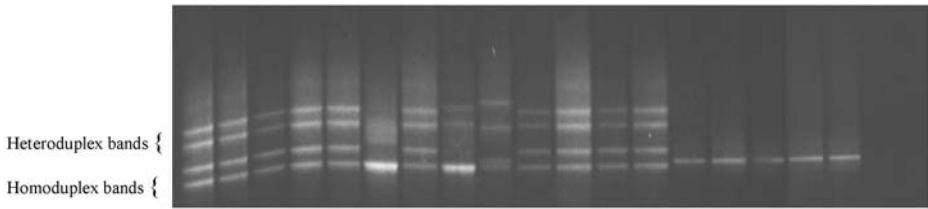


Fig. 3. Southern blot analysis of the HD gene IT15 using *Pst*I-digested genomic DNA, hybridized with the HD-specific DNA probe 4G6P1.7. Sample 1, 2, and 3 are normal. Sample 4 is the 36-repeat control, and sample 5 is a control from an affected person.

can be made. The second polymorphic CCG repeat can be helpful in determining whether the patient has one or two alleles in the normal range.

#### 4.3.2. Southern Blot Alleles

Normal allele sizes vary from 9 to 35 repeats. Normal samples will usually appear as a single band with slight variation in the size. It may also be possible to distinguish two normal bands in some samples. A control sample of 36 repeats must always be included in every analysis. Those samples with two bands, including one that is larger than the 36-repeat control, are classified as having a pathogenic expansion.

#### 4.4. Interpretation of PCR Test Results

1. For DIAGNOSTIC referrals—e.g., patients referred with symptoms suggestive of Huntington's disease:

The presence of two distinct alleles in the normal or intermediate range is usually reported as:

**“This result excludes the diagnosis of Huntington's disease.”** If the laboratory offers a diagnostic test for Dentatorubrel Pallidoluysian Atrophy (DRPLA) (see Note 26), then this may be offered.

The presence of one allele in the normal range and one of 40 or above is usually reported as:

**“This result confirms the diagnosis of Huntington's disease.”**

\*The presence of one allele in the normal range and one allele of 36–39 is unlikely to occur in a patient less than approx 60 yr of age with symptoms suggestive of HD.

2. For patients with a confirmed family history requesting presymptomatic testing:

The presence of two distinct alleles in the normal or intermediate range is usually reported as:

**“This patient has not inherited the expanded CAG repeat present in his/her relative, and the risk of developing HD is therefore extremely low.”**

The presence of one allele in the normal range and one of 40 or above is usually reported as:

**“This patient has an allele of XX repeats which falls into the range known to cause HD. This patient is likely to develop HD at some time in the future.”**

The presence of one allele in the normal range and one allele of 36–39 repeats must be interpreted with caution. Suggested wording may be: **“An allele of this size may show incomplete penetrance with symptoms not becoming apparent until later in life. There is a risk however, of expansion of this allele into the affected range, in subsequent generations.”**

3. Southern blot analysis *should not be used as a front-line test*. Occasionally, analysis using PCR will produce a single band in the normal range. In such cases, Southern analysis is used to distinguish between those patients who either have two repeats of the same size or those who have a primer-site polymorphism causing failure of amplification of one allele. Although not recommended, if an estimation of the size of the expansion is required by this method, then several positive control samples of approx 40, 50, and possibly 70 repeats should be run alongside the test sample, and the size of repeats obtained should be reported with an explanation of the test limitations.
4. When appropriate, genetic counseling should be recommended, and prenatal testing should be offered.

## 5. Notes

1. Since the recognition of the polymorphic 3' C nucleotide in primer HD1, this primer is now synthesized as a 30-mer, excluding this 3' nucleotide. This change does not appear to affect the efficiency of primer annealing.
2. A second forward primer, “Hunt2,” is currently being incorporated into the backup assay to replace HD1. The use of two different primer sets further reduces the risk of error resulting from sequence variation and causing failure of allele amplification. The assay for the HD expansion will therefore involve the amplification of two PCR products with different primer pairs—first, the CAG repeat using HD1/WP2 primers and second, the CAG repeat plus the CCG repeat using the Hunt2/HD2 primers. If both primer pairs are used, a false-negative result will occur, only in the very unlikely event that a patient has a polymorphism in the region of one primer from both pairs.
3. The radioisotope of choice for this application is <sup>32</sup>Phosphorous, because it provides a clear result overnight. <sup>32</sup>Phosphorus is a radioisotope that emits beta radiation of sufficient energy (1.71 MeV) to penetrate water or plastic to a depth of 6 mm and to pass completely through an X-ray film. It has a half-life of 14 d. Gels and membranes do not need to be completely dry, and can be covered in plastic food wrap before they are exposed to the film. The efficiency with which these strong beta particles are detected can be increased by using an intensifying screen, placed behind the X-ray film. Radioactive particles that pass through the film hit the intensifying screen and cause it to emit photons that are captured by the silver halide crystals in the emulsion. This gives an approx fivefold enhancement in the intensity of the autoradiographic image. **Caution:** Specific safety precautions must be followed for the use of this isotope in a laboratory. Consult local regulations.

4. Expand high-fidelity PCR system is composed of an enzyme mix containing thermostable *Taq* polymerase and a proof-reading polymerase.
5. Various standard samples are available from UK labs. Consult the best practice guidelines at [www.cmgs.org](http://www.cmgs.org) and [www.emqn.org](http://www.emqn.org).
6. When glass plates are first used, they should be thoroughly washed in soapy water, then rinsed with water. To ease the removal of the thin acrylamide gel following electrophoresis, the inside surface (next to the gel) of the smaller glass plate is usually treated with Acrylase™ (Stratagene). **Caution:** Acrylase contains acidified ethanol. See safety data sheets. To apply acrylase, spray approx 2 mL onto a clean cloth. Mark the outside of the smaller plate with a piece of colored tape and apply the acrylase evenly to the inside of the plate, using a circular motion and ensuring the entire plate is coated. Allow to dry, then remove the haze with a damp cloth and buff dry. This treatment must be repeated approx every 10 gel runs.
7. For safety reasons, it is recommended that hybridization incubations are carried out using equipment designed specifically for the safe handling of radioisotopes. The procedure can be carried out using a heat-sealed plastic bag or in a perspex box in a temperature-controlled oven. (Care is needed to ensure that plastic bags are completely sealed.) Boxes need to be specially made from a thick perspex rectangle with a central trough approx 3 cm × 10 cm × 1 cm deep. The filters are cut into 2–3-cm × 10-cm strips and labeled for reassembly following hybridization. The strips are layered into a trough containing the radioactive solution. A perspex top is taped onto the box, which is then wrapped in cling-film to minimize leakage during incubation.
8. All test worksheets must be set up following internal quality-control guidelines, including relevant patient information and suitable control samples. This information is available on a UK web site ([www.cmgs.org](http://www.cmgs.org) and [www.emqn.org](http://www.emqn.org)).
9. When calculating volumes for master mix solutions, prepare one extra sample volume for every 10 tubes into which you will be dispensing to allow for pipetting errors—e.g., 11X for 10 tubes.
10. When loading samples onto a vertical polyacrylamide gel with a shark tooth comb, leakage of the sample can occur into the adjacent wells. When this is noted, the contaminated wells must not be used for another sample, and a note must be made on the worksheet of the position of the leaked samples.
11. For UV crosslinking: Air-dry filter. Place in the UV crosslinker on a clean piece of Whatman 3 MM paper, DNA side up, and expose on constant energy setting of 70,000 micro-joules/cm<sup>2</sup> at wavelength 254 nm.
12. T4 polynucleotide kinase (PNK) catalyzes the transfer of the phosphate group from ATP to the 5'-hydroxyl terminus of a DNA molecule, such as a synthetic oligonucleotide or a double-stranded DNA (ds DNA) fragment that has been previously dephosphorylated.
13. Hybrid tube: Position the pipet as far down the center of the tube as possible and then carefully dispense the labeled probe into the solution at the bottom. Replace cap on tube and mix thoroughly by rotating the tube by hand a few times. Replace tube in the hybridization oven as before. Perspex blocks: When using the perspex



blocks, it is advisable to take the membrane strips out of the block, mix the probe with the remaining solution in the block, and then replace the membrane strips, one at a time, to ensure each strip is covered in solution. Replace lid, seal with tape and cover with plastic food wrap.

14. If there is no radioactivity detectable at this stage using a hand-held Geiger counter, it is likely that the oligonucleotide end-labeling step has failed.
15. Silver staining following PAGE is an alternative for laboratories with no facilities for the use of radioisotopes. This technique will stain all products of the amplification, and nonspecific bands may be seen in addition to the amplified CAG repeat. Optimization of the amplification step of the procedure will minimize this risk.
16. If the laboratory has access to an automated DNA sequencer—e.g., ABI 310 or 3100 (ABI, Foster City, CA), a PCR amplification can be carried out in which one of the primers is fluorescently labeled. Fluorescent analysis of the IT15 gene offers another method to distinguish between homozygosity and hemizyosity in patients in whom a single band is obtained following PCR amplification. Such analysis requires the co-amplification of a control sequence of DNA that is known to be present as a single copy on each chromosome. A comparison can be made of the relative doses of each sequence and the ratio calculated. A ratio of 1 : 1 is obtained when the patient is homozygous for a single size allele, and a ratio of 2 : 1 is obtained when amplification of an expanded repeat has failed. Automated fluorescence is particularly useful if the throughput of samples is high.
17. To calculate a volume equivalent to 5  $\mu\text{g}$  of DNA for each test sample. Volume of DNA to be added =  $(5 \times 1000)/\text{concentration of sample in } \mu\text{g/mL}$ . OR The volume of DNA required for a 5- $\mu\text{g}$  digest, at various DNA concentrations is given in **Table 2**.
18. For DNA samples that are resistant to restriction enzyme digestion add BSA (2  $\mu\text{L}$  of a 10 mg/mL stock solution) and spermidine (0.8  $\mu\text{L}$  of 100 mM stock solution) to the restriction enzyme digestion.
19. The gel electrophoresis can be carried out overnight. In this case, it is best run in 1X TBE buffer at 40 V. Buffer recirculation is usually not necessary for the separation of DNA restriction fragments, as adequate size fractionation is achieved by overnight electrophoresis.
20. When troubleshooting a failed hybridization, precipitate the radiolabeled probe by adding 15  $\mu\text{L}$  of 7.5 M  $\text{NH}_4\text{OAc}$  and 90  $\mu\text{L}$  of 100% ethanol, then chill at  $-20^\circ\text{C}$  for at least 1 h. Centrifuge in a mini-centrifuge at 10,500g for 15 min to precipitate the DNA, then carefully discard the supernatant. The probe pellet will not be visible to the eye, so always set the tubes in the microfuge with the hinge to the outside. The pellet will then be at the bottom of the tube on the hinge side. Resuspend the pellet in 200 mL T.E. Measure the specific activity of the labeled probe in a benchtop counter or by using a hand-held Geiger counter).
21. Desired probe specific activity:  $10^8$  cpm/mg probe. Probe concentration in the hybridization mixture:  $10^6$  cpm/mL (10–15 ng/mL).
22. If the background is high, then additional washes at increased stringency are performed. If the background is clear and the bands are weak, then a new film is exposed



for a longer period up to 7–10 d. (Beyond 10 d, the emission of the isotope is unlikely to be strong enough to produce a denser band). If the bands cannot be interpreted at this point, re-hybridization with a fresh aliquot of probe is recommended.

23. Intermediate alleles were found in approx 1.5–2% of the general population in the U.S. survey (**14,15**). These have not been associated convincingly with the HD phenotype; however, alleles in this size range have demonstrated mutability in male transmission, and meiotic instability has been demonstrated in sperm for alleles in this group.
24. Reduced-penetrance alleles have been clinically associated with the HD phenotype (**4**) and pathologically (**2**). However, these alleles are not always penetrant, and some individuals age >70 yr with 36–39 repeats have been reported to be free of HD symptoms.
25. Trinucleotide-repeat alleles do not appear as discrete DNA bands on polyacrylamide gels, but as an allelic band accompanied by one or two or more stutter bands. This is because during the replication of the trinucleotide repeat in the PCR reaction, slippage can occur, which leads to omission of multiples of the repeat. This can be improved by utilizing a Taq polymerase with proof-reading ability. Using the radiolabeled method of analysis, the stutter bands are usually clearly visible, providing a ladder of repeats that usually extends from the normal allele to an allele in the abnormal range if present, thereby facilitating accurate sizing.
26. The clinical spectrum seen in Huntington's disease may overlap with that seen in other genetic conditions. For symptomatic individuals who have two normal size IT15 repeats, most laboratories offer analysis for DRPLA; however, recent publications indicate that it may be necessary to consider extending the testing to include the analysis of the gene for ferritin light chain (**16**), prion disease (**17**), and genes in which mutations have been found to give rise to Huntington phenocopy (**18**).

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## Hematological Applications

### *Hemoglobinopathies*

**John M. Old**

#### **1. Introduction**

The hemoglobinopathies are a diverse group of inherited recessive disorders that include the thalassemias and sickle-cell disease. These were the first genetic diseases to be characterized at the molecular level, and consequently have been used as a prototype for the development of new techniques of mutation detection. There are now many different polymerase chain reaction (PCR)-based techniques that can be used to diagnose the globin gene mutations, including dot blot analysis, reverse dot blot analysis, the amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis (DGGE), mutagenically separated PCR, gap PCR, and restriction endonuclease analysis (1,2). Each method has its advantages and disadvantages, and the particular one chosen by a laboratory to diagnose point mutations in the various globin genes depends on the technical expertise available in the diagnostic laboratory as well as the type and variety of the mutations that are likely to be encountered in the individuals being screened.

#### **1.1. Diagnostic Approaches**

The main diagnostic approaches for the PCR diagnosis of the hemoglobinopathies are listed in **Table 1**. The techniques most often used in this laboratory are gap-PCR, ARMS-PCR, and restriction endonuclease (RE) analysis of amplified product. Detailed protocols for each of these techniques are presented in this chapter. The alternative method often used for the diagnosis of hemoglobin mutations—allele-specific oligonucleotide (ASO) hybridization by

**Table 1**  
**DNA Diagnosis of the Hemoglobinopathies**

Disorder and mutation type	Diagnostic method
$\alpha^0$ -thalassemia	Gap-PCR, Southern blotting
$\alpha^+$ -thalassemia:	
deletion	Gap-PCR, Southern blotting
non-deletion	ASO, RE, DGGE
$\beta$ -thalassemia:	
deletion	Gap-PCR
non-deletion	ASO, ARMS, DGGE
$\delta\beta$ -thalassemia	Gap-PCR, Southern blotting
HPFH	
deletion	Gap-PCR, Southern blotting
non-deletion	ASO, ARMS, RE, DGGE
Hb Lepore	Gap-PCR
Hb S	ASO, ARMS, RE
Hb C	ASO, ARMS
Hb E	ASO, ARMS, RE
Hb D Punjab	ASO, ARMS, RE
Hb O Arab	ASO, ARMS, RE
Hb variants	RT-PCR and DNA sequencing

dot blotting or reverse dot blotting—is not covered in this chapter, but detailed protocols may be found elsewhere (3).

The hemoglobinopathies are regionally specific, and each population has a unique combination of abnormal hemoglobins and thalassemia disorders. The spectrum of mutations and mutation frequencies has been published for most populations. Therefore, knowledge of the ethnic origin of a patient simplifies the diagnostic strategy, enabling a quick identification of the underlying defects in most cases.

### 1.2. $\alpha$ -Thalassemia

Gap-PCR (amplification across the breakpoints of a deletion) provides a quick diagnostic test for  $\alpha^+$ -thalassemia and  $\alpha^0$ -thalassemia deletion mutations, but requires careful application for prenatal diagnosis. Most of the common  $\alpha$ -thalassemia alleles that result from gene deletions can be diagnosed by gap-PCR. Primer sequences have now been published for the diagnosis of five  $\alpha^0$ -thalassemia deletions and two  $\alpha^+$ -thalassemia deletions (4–7), as listed in **Table 2**. The  $\alpha^0$ -thalassemia deletions diagnosable by PCR are: the

**Table 2**  
**Thalassemia Deletion Mutations That Have Been Diagnosed by Gap-PCR**

Disorder	Deletion mutation	Reference
$\alpha^0$ -thalassemia	$_{-SEA}$	4
	$_{-MED}$	4
	$-(\alpha)^{20.5}$	4
	$_{-FIL}$	6,7
	$_{-THAI}$	6,7
$\alpha^+$ -thalassemia	$-\alpha^{3.7}$	5
	$-\alpha^{4.2}$	5
$\beta^0$ -thalassemia	290-bp deletion	22
	532-bp deletion	23
	619-bp deletion	24
	1393-bp deletion	25
	1605-bp deletion	26
	3.5-kb deletion	27
	10.3-kb deletion	28
$(\delta\beta)^0$ thalassemia	45-kb deletion	29
	Hb Lepore	18
	Spanish	18
	Sicilian	18
	Vietnamese	18
$(\Lambda\gamma\delta\beta)^0$ thalassemia	Macedonian/Turkish	18
	Indian	18
HPFH	Chinese	18
	HPFH1 (African)	18
	HPFH2 (Ghanaian)	18
	HPFH3 (Indian)	18

$_{-SEA}$  allele, found in Southeast Asian individuals; the  $_{-MED}$  and  $-(\alpha)^{20.5}$  alleles found in Mediterranean individuals; the  $_{-FIL}$  allele, found in Filipino individuals, and finally, the  $_{-THAI}$  allele, found in Thai individuals. The two  $\alpha^+$ -thalassemia deletion mutations are the 3.7-kb and the 4.2-kb single  $\alpha$ -gene deletion mutations, designated  $-\alpha^{3.7}$  and  $-\alpha^{4.2}$ . The former is found in African, Mediterranean, Asian, and Southeast Asian populations, and the latter is found in Southeast Asian and Pacific populations. However, it is good practice to screen for both deletions in any individual. The first gap PCR assays were subject to technical failure, but the more recently published primers and conditions (6,7) result in a more robust assay (*see Note 1*). **Figures 1** and **2** show

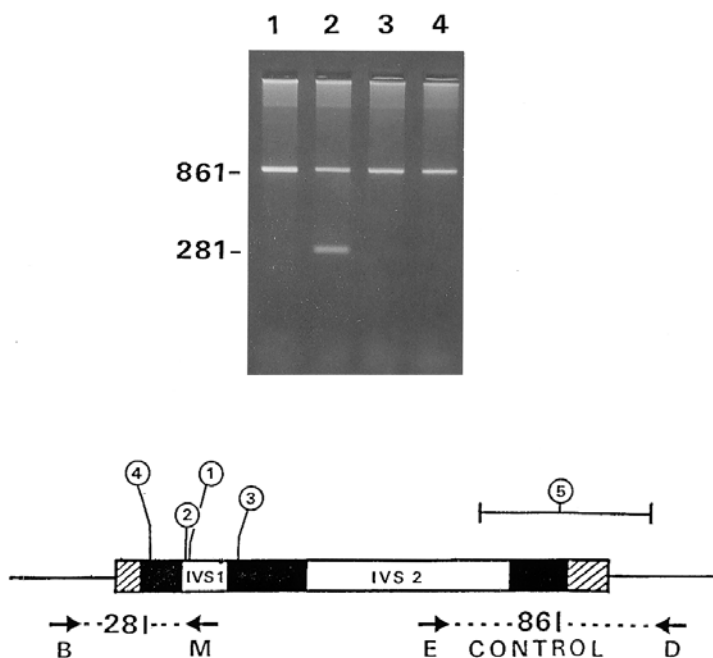


Fig. 1. Ethidium bromide-stained gel showing the screening of a DNA sample for the five common Asian Indian  $\beta$ -thalassemia mutations by ARMS-PCR. The five mutations screened for individually are: track1, IVSI-5 (G $\rightarrow$ C); 2, IVSI-1 (G $\rightarrow$ T); 3, codon 41/42 (-TCTT); 4, codon 8/9 (C $\rightarrow$ T); all tracks, 619 bp deletion. The results show the patient carries the mutation IVSI-1 (G $\rightarrow$ T). The control primers D and E produce an 861-bp fragment with normal DNA, plus an abnormal fragment of 242 bp in all tracks if the patient carries the 619-bp deletion mutation. The primers used are listed in **Table 5**. The diagram shows the  $\beta$ -globin gene position of the five common mutations screened for in the gel shown in the figure.

the results of screening for the five common  $\alpha^0$  and the two common  $\alpha^+$ -thalassemia mutations with the more robust primers.

The other  $\alpha^0$  and  $\alpha^+$ -thalassemia mutations cannot be diagnosed by PCR because their breakpoint sequences have not been determined. These deletion mutations are diagnosed by the Southern blotting technique, using  $\xi$ -gene and  $\alpha$ -gene probes. This approach is still very useful because it permits the diagnosis of  $\alpha$ -thalassemia deletions and  $\alpha$ -gene rearrangements (the triple and quadruple  $\alpha$ -gene alleles) in a single test (8). The characteristic abnormal fragments used in this laboratory for the diagnosis of the commoner  $\alpha$ -thalassemia deletions are listed in **Table 3**.

**Table 3**  
**The Diagnosis of  $\alpha$ -Thalassemia Alleles by Southern Blotting**

Allele	Restriction enzyme/gene probe				
	<i>Bam</i> HI / $\alpha$	<i>Bg</i> III / $\alpha$	<i>Bam</i> HI / $\xi$	<i>Bg</i> III / $\xi$	<i>Sst</i> I / LO
$\alpha\alpha$	14	12.6 7.4	10–11.3 5.9	12.6 or 5.2 10–11.3	5.0
$\alpha\alpha\alpha$	<u>18</u>	12.6 7.4	10–11.3 5.9	12.6 or <u>16</u> 10–11.3	5.0
$\alpha\alpha\alpha\alpha$	<u>22</u>	12.6 7.4	10–11.3 5.9	12.6 or <u>20</u> 10–11.3	5.0
$-\alpha^{3.7}$	<u>10.3</u>	<u>16</u>	10–11.3 5.9	<u>16</u> 10–11.3	5.0
$-\alpha^{4.2}$	<u>9.8</u>	<u>8.0</u> 7.4	10–11.3 5.9	10–11.3 <u>8.0</u>	5.0
$-(\alpha)^{20.5}$	<u>4.0</u>	<u>10.8</u>	5.9	<u>10.8</u>	5.0
$_{-MED}$	None	None	5.9	<u>13.9</u>	5.0
$_{-SEA}$	None	None	<u>20</u> 5.9	<u>10.5</u>	5.0
$_{-SA}$	None	None	5.9	<u>7.0</u>	5.0
$_{-BRIT}$	None	None	5.9	<u>7.5</u>	5.0
$_{-THAI}$	None	None	None	None	<u>8.0</u>
$_{-FIL}$	None	None	None	None	<u>7.4</u>

Fragment sizes are given in kilobase pairs. Characteristic abnormal fragments are underlined.

$\alpha^+$ -Thalassemia is also caused by point mutations in one of the two  $\alpha$ -globin genes. These non-deletion alleles can be detected by PCR using a technique of selective amplification of each  $\alpha$ -globin gene followed by a general method of mutation analysis such as denaturing gradient-gel electrophoresis (DGGE) (9) or DNA sequence analysis (10). Several of the non-deletion mutations alter a restriction enzyme site and may be diagnosed by selective amplification and restriction endonuclease analysis in a similar manner to that reported for the mutation which gives rise to the unstable  $\alpha$ -globin chain variant Hb Constant Spring (11).

### 1.3. $\beta$ -Thalassemia

The  $\beta$ -thalassemias are caused by a very heterogeneous group of defects with more than 170 different mutations characterized to date (12). The majority of the defects are single-nucleotide substitutions, insertions, or deletions. Only 13 large gene deletions have been identified, and eight of these can be diagnosed

**Table 4**  
**The Distribution of the Common  $\beta$ -Thalassemia Mutations Expressed as Percentage of Gene Frequencies of the Total Number of Thalassemia Chromosomes Studied**

Mutation	Mediterranean			India		Chinese		African
	<i>Italy</i>	<i>Greece</i>	<i>Turkey</i>	<i>Pakistan</i>	<i>India</i>	<i>China</i>	<i>Thailand</i>	<i>African-American</i>
-88 (C→T)					0.8			21.4
-87 (C→G)	0.4	1.8	1.2					
-30 (T→A)			2.5					
-29 (A→G)						1.9		60.3
-28 (A→G)						11.6	4.9	
CAP+1 (A→C)					1.7			
CD5 (-CT)		1.2	0.8					
CD6 (-A)	0.4	2.9	0.6					
CD8 (-AA)		0.6	7.4					
CD8/9 (+G)				28.9	12.0			
CD15 (G→A)				3.5	0.8			0.8
CD16 (-C)				1.3	1.7			
CD17 (A→T)						10.5	24.7	
CD24 (T→A)								7.9
CD39 (C→T)	40.1	17.4	3.5					
CD41/42 (-TCTT)				7.9	13.7	38.6	46.4	
CD71/72 (+A)						12.4	2.3	
IVSI-1 (G→A)	4.3	13.6	2.5					
IVSI-1 (G→T)				8.2	6.6			
IVSI-5 (G→C)				26.4	48.5	2.5	4.9	
IVSI-6 (T→C)	16.3	7.4	17.4					
IVSI-110 (G→A)	29.8	43.7	41.9					
IVSII-1 (G→A)	1.1	2.1	9.7					
IVSII-654 (C→T)						15.7	8.9	
IVSII-745 (C→G)	3.5	7.1	2.7					
619-bp deletion				23.3	13.3			
Others	4.1	2.2	9.7	0.5	0.9	6.8	7.9	10.6

CD, codon; IVS, intervening sequence; bp, basepairs.

by gap PCR, as listed in **Table 2**. The other types of mutations can be diagnosed by a variety of methodologies, but the strategy for identifying  $\beta$ -thalassemia mutations remains the same. The mutations are regionally specific, and each at-risk population has a few common mutations together with a larger, variable number of rare ones (**Table 4**). The strategy depends on knowledge of the spec-



trum of common and rare mutations in the ethnic group of the individual being screened. The common ones are analyzed first using a PCR method designed to detect specific mutations simultaneously. This approach will identify the mutation in more than 80% of cases for most ethnic groups. A further screening of the known rare mutations will identify the defect in another 10–15% of cases if necessary. Mutations that remain unidentified at this stage are characterized by DNA sequencing.

The first PCR diagnostic method to be developed and gain widespread use was the hybridization of ASOs to amplified DNA bound to nylon membrane by dot-blotting (13). Although still in use, the method is limited by the need for separate hybridization steps to test for multiple mutations. This problem has been overcome by the development of the reverse dot-blotting technique, in which amplified DNA is hybridized to a panel of mutation-specific probes fixed to a nylon strip. This technique is compatible with the optimum strategy for screening  $\beta$ -thalassemia mutations, using a panel of the commonly found mutations for the first screening and a panel of rare ones for the second screen (14).

The technique used by this laboratory and described here is the ARMS system. This technique fits the main requirements of a PCR technology—speed, cost, convenience, and the ability to test for multiple mutations simultaneously, as it provides a quick cheap screening method without any form of labeling of primers or amplified DNA. The simplest way is to screen for mutations with simultaneous PCR assays, although the multiplexing of ARMS primers in a single PCR assay is possible (15). **Figure 1** shows the results of screening a patient's DNA sample for the five common Asian Indian  $\beta$ -thalassemia mutations.

The most widely used indirect method to characterize  $\beta$ -thalassemia mutations is DGGE (16). The technique detects at least 90% of  $\beta$ -thalassemia mutations by producing a shifted band pattern to normal, and provides an alternative approach to ASO probes or ARMS in countries in which a very large spectrum of  $\beta$ -thalassemia mutations occurs (17).

#### 1.4. $\delta\beta$ -Thalassemia and HPFH

$\delta\beta$ -Thalassemia and the hereditary persistence of fetal hemoglobin (HPFH) disorders result from large gene deletions that affect both the  $\beta$ - and  $\delta$ -globin genes. Restriction enzyme mapping has enabled the characterization of more than fifty different deletions starting at different points between the  $\gamma$ -gene and the  $\delta$ -gene and extending up to 100 kb downstream of the  $\beta$ -globin gene. In two cases, the Macedonian/Turkish ( $\delta\beta$ )<sup>o</sup>-thalassemia gene and the Indian ( $\Delta\gamma\delta\beta$ )<sup>o</sup>-thalassemia gene, the mutation is a complex rearrangement consisting of an inverted DNA sequence flanked by two deletions. A small number of these deletions have had their breakpoint sequences characterized, and these can be diag-

nosed by gap-PCR (18). Gap-PCR can also be used for the diagnosis of the Hb Lepore, created by a deletion of the DNA sequence between the  $\delta$ - and  $\beta$ -globin genes. Hb Lepore is the product of the  $\delta\beta$  fusion gene, and is associated with a severe  $\beta$ -thalassemia phenotype. All the deletion mutations currently diagnosable by gap-PCR are listed in **Table 2**; the others can only be diagnosed by the identification of characteristic breakpoint fragments with Southern blot analysis.

### 1.5. Hb Variants

More than 700 hemoglobin variants have been described to date, and most of those were identified by protein analysis and have never been characterized at the DNA level. Positive identification at the DNA level is achieved by selective globin gene amplification and DNA sequence analysis. However, the clinically important variants—Hb S, Hb C, Hb E, Hb D Punjab, and Hb O Arab—can be diagnosed by simpler DNA analysis techniques. Sickle-cell disease is caused by homozygosity for Hb S, and also—in varying degrees of severity—from the interaction of Hb S with Hb C, Hb D Punjab, Hb O Arab, and  $\beta$ -thalassemia trait. All these variants can be diagnosed by ASO hybridization, the ARMS technique, or—for all except Hb C—by restriction endonuclease digestion of amplified product. The sickle-cell gene mutation abolishes a *Dde* I recognition site at codon 6, and diagnosis by *Dde* I digestion of amplified product remains the simplest method of DNA analysis for sickle-cell disease. Similarly, the mutations that give rise to Hb D Punjab and Hb O Arab abolish an *Eco*R I site at codon 121. However, the Hb C mutation at codon 6 does not abolish the *Dde* I site, and can be diagnosed by other methods. Hb E interacts with  $\beta$ -thalassemia trait to produce a clinical disorder of varying severity, ranging from thalassemia intermedia to transfusion dependent thalassemia major. The Hb E mutation can be diagnosed by ASO hybridization, ARMS, or restriction endonuclease analysis, as the mutation abolishes a *Mnl* I site in the  $\beta$ -globin gene sequence.

## 2. Materials

1. For ARMS PCR, the buffer used is the standard PCR buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at room temperature), 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/mL gelatin. A 10X stock buffer can be prepared by adding together 0.5 mL of 1 M Tris-HCl (pH 8.3 at room temperature), 1.25 mL of 2 M KCl, 75  $\mu$ L of 1 M MgCl<sub>2</sub>, 5 mg gelatin, and 3.275 mL of distilled water. The stock buffer is heated at 37°C until the gelatin dissolves, and then frozen in aliquots.
2. For gap PCR, the reaction buffer (10X) recommended for the particular pair of primers being used is required. The buffer for  $\alpha$ -thalassemia primers also includes 0.5 M betaine.

3. The stock deoxynucleotide mixture contains each deoxynucleotide 5' triphosphate (dNTP) at 1.25 mM. Add together 50  $\mu$ L of a 100-mM solution of each dNTP (as purchased) and 3.8 mL of distilled water. The 1.25-mM dNTP stock solution should be stored in frozen aliquots.
4. The PCR reaction stock solution (4 mL) is made of 0.5 mL of 10X buffer, 0.8 mL 1.25 mM dNTP stock solution, and 2.7 mL distilled water.
5. Dilute aliquots of primer stock solutions to make a working solution of 1 optical density (OD) U/mL and store frozen.
6. Taq polymerase: AmpliTaq Gold (PE Biosystems) is recommended by the author.

### 3. Methods

#### 3.1. Gap-PCR

Gene deletion mutations in the  $\beta$ -globin gene cluster may be detected by PCR using two primers that are complementary to the sense and anti-sense strand in the DNA regions that flank the deletion. For small deletions of less than 1 kb in size, the primer pair will generate two products, the smaller fragment arising from the deletion allele. For large deletions, the distance between the two flanking primers is too great to amplify the normal allele, and product is only obtained from the deletion allele. In these cases, the normal allele is detected by amplifying across one of the breakpoints, using a primer that is complementary to the deleted sequence and one that is complementary to the flanking DNA.

1. Set up the reaction mixture to a final vol of 22  $\mu$ L in a 0.5-mL tube with the following components as required: 1  $\mu$ L genomic DNA (100 ng/ $\mu$ L), 1  $\mu$ L of forward primer-flanking sequence (10 pmol/ $\mu$ L), 1  $\mu$ L reverse primer-flanking sequence (10 pmol/ $\mu$ L), 1  $\mu$ L of primer-deleted sequence (10 pmol/ $\mu$ L), 1  $\mu$ L of primer-inverted sequence (10 pmol/ $\mu$ L), 2.5  $\mu$ L of 1.25 mM (dNTP mixture), 2.3  $\mu$ L of the reaction buffer (10X) as recommended for the primers, sterile dH<sub>2</sub>O to a final vol of 22  $\mu$ L. The reaction mixture for the  $\alpha$ -thalassemia primers also contains 0.5 M betaine: add 2.5  $\mu$ L of 5 M betaine (Sigma).
2. Overlay with 25  $\mu$ L of mineral oil (unnecessary if a PCR block with heated lid is used).
3. Prepare enzyme mixture: 0.2  $\mu$ L reaction buffer (10X), 0.1  $\mu$ L Ampli Taq (5 U/ $\mu$ L) (PE Biosystems) for the  $\beta$ -gene primers, 0.1  $\mu$ L Platinum Taq (5 U/ $\mu$ L) (Life Technologies) for the  $\alpha$ -gene primers, and 2.7  $\mu$ L sterile dH<sub>2</sub>O to a final volume of 3  $\mu$ L.
4. Mix enzyme mixture and hold on ice.
5. Place reaction mixtures in thermal cycler and perform one cycle as follows, adding 3  $\mu$ L of the enzyme mix after 2 min of the 94°C denaturation step: 4 min at 94°C/1 min at 55–65°C (as recommended for the particular gap PCR reaction)/1.5 min at 72°C.

6. Continue for 33 cycles with the following steps per cycle: 1 min at 94°C/1 min at 55–65°C (as recommended for the particular gap PCR reaction)/1.5 min at 72°C.
7. Finish with one cycle as follow: 1 min at 94°C/1 min at 55–65°C (as recommended for the particular gap PCR reaction)/10 min at 72°C.
8. Hold at 15°C until the gel electrophoresis step.
9. Remove tubes from thermal cycler and add 5  $\mu$ L of loading buffer (15% ficoll/0.05% bromophenol blue). Mix and centrifuge.
10. Load a 20- $\mu$ L aliquot onto a 1–3% agarose gel (depending on expected fragment sizes) and run at 100V for 45 mins in Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (89 mM Tris-borate, 89 mM boric acid, 10 mM EDTA 0 pH 8.0).
11. Stain gel in ethidium bromide solution (0.5  $\mu$ g/mL) for 15–30 min, visualize bands on a UV light box (312 nm), and photograph with an electronic camera system or a Polaroid CU-5 camera fitted with an orange filter (e.g., Wratten 22A).

### 3.2. ARMS PCR

The ARMS technique for detecting known point mutations was first described by Newton et al. (19). It has been developed for the diagnosis of all the common  $\beta$ -thalassemia mutations and many of the rare ones (8,20). The technique is based on the principle of allele-specific priming of the PCR process—i.e., a specific primer will only permit amplification to take place when its 3' terminal nucleotide matches with its target sequence. Only strong 3' terminal mismatches (C-C, G-A and A-A) reduce priming efficiency to zero or below  $-5\%$  (21). To prevent amplification with weak mismatches, a further mismatch with the target sequence is introduced at the second, third, or fourth nucleotide from the 3' end of the primer. As a general rule, if the 3' terminal mismatch is a weak one, a strong secondary mismatch is engineered. If it is a strong one, a weak secondary mismatch is introduced.

The mutation-specific ARMS primers used in this laboratory to diagnose the 25 most common  $\beta$ -thalassemia mutations, plus the hemoglobin variants Hb S, Hb C and Hb E, are listed in **Table 5**. All are 30 bases long so that they can all be used at a single high annealing temperature (65°). Primers for the diagnosis of the normal alleles for many of these mutations are listed in **Table 6**. These are required when both partners of a couple requesting prenatal diagnosis of  $\beta$ -thalassemia carry the same mutation.

A typical ARMS test for a single mutation consists of two amplifications in the same reaction mixture using the same genomic DNA as substrate. One amplification product results from the specific ARMS primer and its primer pair (when the mutation is present in the genomic DNA), and the other amplification results from two primers that generate a control fragment in all cases. The generation of control product indicates that the reaction mixture and thermal cycler are working optimally (*see Notes 2–6*).

**Table 5**  
**ARMS-PCR Primer Sequences Used for the Detection**  
**of the Common  $\beta$ -Thalassemia Mutations**

Mutation	Oligonucleotide sequence (5'-3')	Second Primer	Product Size (bp)	Altered Restriction Site
-88 (C→T)	TCACCTTAGACCTCACCCCTGTGGAGCCTCAT	A	684	+ <i>FokI</i>
-87 (C→G)	CACTTAGACCTCACCCCTGTGGAGCCACCCG	A	683	- <i>AvrII</i>
-30 (T→A)	GCAGGGAGGGCAGGAGCCAGGGCTGGGGAA	A	626	
-29 (A→G)	CAGGGAGGGCAGGAGCCAGGGCTGGGTATG	A	625	+ <i>NlaIII</i>
-28 (A→G)	AGGGAGGGCAGGAGCCAGGGCTGGGCTTAG	A	624	
CAP+1(A→G)	ATAAGTCAGGGCAGAGCCATCTATTTGGTTC	A	597	
CD5 (-CT)	TCAAACAGACACCATGGTGCACCTGAGTCG	A	528	- <i>DdeI</i>
CD6 (-A)	CCCACAGGGCAGTAACGGCAGACTTCTGCC	B	207	- <i>DdeI</i>
CD8 (-AA)	ACACCATGGTGCACCTGACTCCTGAGCAGG	A	520	
CD8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCAGACC	B	225	
CD15 (G→A)	TGAGGAGAAGTCTGCCGTACTGCCCAGTA	A	500	
CD16 (-C)	TCACCACCAACTTCATCCACGTTACGTTTC	B	238	
CD17 (A→T)	CTCACCACCAACTTCATCCACGTTACGCTA	B	239	+ <i>MaeI</i>
CD24 (T→A)	CTTGATAACCAACTGCCCAGGGCCTCTCCT	B	262	
CD39 (C→T)	CAGATCCCCAAAGGACTCAAAGAACCTGTA	B	436	+ <i>MaeI</i>
CD41/42 (-TCTT)	GAGTGGACAGATCCCCAAAGGACTCAACCT	B	439	
CD71/72 (+A)	CATGGCAAGAAAGTGCTCGGTGCCCTTAAAG	C	241	
IVSI-1 (G→A)	TTAAACCTGTCTTTGTAACCTTGATACCGAT	B	281	- <i>BspMI</i>
IVSI-1 (G→T)	TTAAACCTGTCTTTGTAACCTTGATACGAAA	B	281	- <i>BspMI</i>
IVSI-5 (G→C)	CTCCTTAAACCTGTCTTTGTAACCTTGTTAG	B	285	
IVSI-6 (T→C)	TCTCCTTAAACCTGTCTTTGTAACCTTCATG	B	286	+ <i>SfaNI</i>
IVSI-110 (G→A)	ACCAGCAGCCCTAAGGGTGGGAAAAATAGAGT	B	419	
IVSII-1 (G→A)	AAGAAAACATCAAGGGTCCCATAGACTGAT	B	634	- <i>HphI</i>
IVSII-654 (C→T)	GAATAACAGTGATAATTTCTGGGTAAACGT*	D	829	
IVSII-745 (C→G)	TCATATTGCTAATAGCAGCTACAATCGAGG*	D	738	+ <i>RsaI</i>
$\beta^S$ CD6 (A→T)	CCCACAGGGCAGTAACGGCAGACTTCTGCA	B	207	- <i>DdeI</i>
$\beta^C$ CD6 (G→A)	CCACAGGGCAGTAACGGCAGACTTCTCGTT	B	206	
$\beta^E$ CD26 (G→A)	TAACCTTGATACCAACTGCCCAGGGCGTT	B	236	- <i>MnII</i>

The above primers (5'-3') are coupled as indicated with either primer A, CCCCTTCTATGACATGAACCTTAA; B, ACCTCACCCCTGTGGAGCCAC; C, TTCGTCTGTTCCCATTTCTAAACT; or D, GAGTCAAGGCTGAGAGATGCAGGA. The control primers used were primers D plus E: CAATGTATCATGCCTCTTTGCACC (which yield a 861-bp product) for all the above mutation specific ARMS primers except the two marked \*. Control primers used with these two are the  $\gamma$ -*HindIII* RFLP primers (8).

1. Prepare a reaction mixture (4 mL) comprised of: 0.5 mL of 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/mL gelatin); 1.25 mL of 1.35 mM dNTP mixture; 2.65 mL of sterile distilled water.
2. Pipet 20  $\mu$ L of PCR reaction mixture into a 0.5- $\mu$ L tube.

**Table 6**  
**Primer Sequences Used for the Detection of the Normal DNA Sequence by the Allele-Specific Priming Technique**

Mutation	Oligonucleotide Sequence (5'–3')	Second Primer	Product Size (kb)
–87 (C→G)	CACTTAGACCTCACCTGTGGAGCCACCCC	A	683
CD5 (–CT)	CAAACAGACACCATGGTGCACCTGACTCCT	A	528
CD8 (–AA)	ACACCATGGTGCACCTGACTCCTGAGCAGA	A	520
CD8/9 (+G)	CCTTGCCCCACAGGGCAGTAACGGCACACT	B	225
CD15 (G→A)	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	A	500
CD39 (C→T)	TTAGGCTGCTGGTGGTCTACCCTTGGTCCC	A	299
CD41/42 (–TCTT)	GAGTGGACAGATCCCCAAAGGACTCAAAGA	B	439
IVSI-1 (G→A)	TTAAACCTGTCTTGTAACCTTGATACCCAC	B	281
IVSI-1 (G→T)	GATGAAGTTGGTGGTGAGGCCCTGGGTAGG	A	455
IVSI-5 (G→C)	CTCCTTAAACCTGTCTTGTAACCTTGTTAC	B	285
IVSI-6 (T→C)	AGTTGGTGGTGAGGCCCTGGGCAGGTTGGT	A	449
IVSI-110 (G→A)	ACCAGCAGCCTAAGGGTGGGAAAATACACC	B	419
IVSII-1 (G→A)	AAGAAAACATCAAGGGTCCCATAGACTGAC	B	634
IVSII-654 (C→T)	GAATAACAGTGATAATTTCTGGGTTAACGC	D	829
IVSII-745 (C→G)	TCATATTGCTAATAGCAGCTACAATCGAGC	D	738
β <sup>S</sup> CD6 (A→T)	AACAGACACCATGGTGCACCTGACTCGTGA	A	527
β <sup>F</sup> CD26 (G→A)	TAACCTTGATACCAACCTGCCCAGGGCGTC	B	236

See **Table 4** legend for details of primers A–D and control primers.

3. Add 1 μL of each primer (1 OD U/mL).
4. Add 0.05 μL of Taq DNA polymerase (5 U/μL).
5. When more than one test is being performed, a primer and the enzyme can be mixed together in a separate tube before addition to the reaction mix. This decreases pipetting errors because larger quantities are used.
6. Add 1 μL of genomic DNA (100 ng/μL).
7. Overlay with 25 μL of mineral oil.
8. Mix, centrifuge, and place in a thermal cycler.
9. Amplify for 25 cycles as follows: 1 min at 94°C/1 min at 65°C/1.5 min at 72°C with a final extension period of 3 min at 72°C following the 25th cycle.
10. Remove tubes from thermal cycler and add 5 μL of loading buffer (15% ficoll/0.05% bromophenol blue). Mix and centrifuge.
11. Load a 20 μL aliquot onto a 3% agarose gel and run at 100V for approx 45 min in TBE (see protocol 1).
12. Stain with ethidium bromide and visualize bands as described in the gap-PCR protocol.

### 3.3. Restriction Enzyme Digestion

A small number of the β-thalassemia mutations create or abolish a RE recognition site in the globin gene sequence. Provided that the enzyme is commercially

available (this is not always the case), and that there is not another site too close to the mutation, the loss or creation of a site can be used to diagnose the presence or absence of the mutation. This is useful for the diagnosis of a few of the common  $\beta$ -thalassemia mutations, as listed in **Table 4**, but the main use of this PCR technique is for the diagnosis of the clinically important Hb variants Hb S, Hb D Punjab, and Hb O Arab. The primer sequences used in my laboratory for diagnosing these Hb variants are listed in **Table 7**. When possible, the amplified product should include a second site for the appropriate restriction enzyme. This site will act as a control for the digestion reaction, as it should be fully cleaved in product from both the normal and mutant DNA alleles. This is possible for the Hb S and Hb E mutations, but not for Hb O Arab and Hb D Punjab, for which the flanking *Eco*RI sites are too far away from the one in codon 121.

1. To 1 0.5-mL tube, add the following: 20  $\mu$ L PCR reaction mixture (as detailed in protocol 2); 1  $\mu$ L each primer; 1  $\mu$ L genomic DNA (100 ng/ $\mu$ L); 2  $\mu$ L sterile dH<sub>2</sub>O; 0.05  $\mu$ L of AmpliTaq DNA polymerase (5 U/ $\mu$ L)
2. Overlay with 25  $\mu$ L of mineral oil.
3. Place in thermal cycler and perform 30 cycles of: 1 min at 94°C/1 min at 65°C/1.5 min at 72°C with a final period at 72°C for 3 min after the last cycle.
4. Remove tubes and add 5–10 U of the appropriate restriction enzyme, plus 2  $\mu$ L of the corresponding 10X buffer.
5. Incubate at 37°C for a minimum of 1 h.
6. Add loading buffer, mix, and spin as in protocol 1.
7. Load 20  $\mu$ L aliquot onto a 3% agarose gel consisting of 50% Nusieve GTC agarose and 50% ordinary agarose (Flowgen).
8. Carry out electrophoresis, stain, and photograph as in protocol 1.

#### 4. Notes

1. Gap PCR seems to work reasonably well for amplifying deletion mutations in the  $\beta$ -globin gene cluster. However amplification of deletions in the  $\alpha$ -gene cluster is technically more difficult, possibly because of the high GC content of the  $\alpha$ -globin gene-cluster sequence. Our experience in this laboratory has shown that some of the first primer pairs that were published were unreliable, occasionally resulting in unpredictable reaction failures caused by allele drop out, especially when the normal and mutant specific primers were multiplexed together. However, the recently published multiplex primers seem to give more robust and reproducible results. The addition of betaine to the reaction mixture and the use of Platinum Taq DNA polymerase (which has been developed for automatic “hot start” amplification of problematic or GC-rich templates) are key features of their success. The technique is useful for screening for a particular  $\alpha^0$ -thalassemia deletion mutation, but because of the problem of potential allele-drop out, prenatal diagnosis results should be confirmed by Southern blot analysis. The new primers are designed for a multiplex screening test, although in this laboratory they are still used in pairs to test for individual mutations.

**Table 7****Oligonucleotide Primers for the Detection of  $\beta^S$ ,  $\beta^E$ ,  $\beta^D$  Punjab, and  $\beta^O$  Arab Mutations as RFLPs**

Mutation and affected RE site	Primer Sequences (forward and reverse, 5'-3')	Annealing Temperature (°C)	Product size, bp	Absence of site, bp	Presence of site, bp
$\beta^S$ : CD 6 (A→T) (Loss of <i>DdeI</i> site)	ACCTCACCCCTGTGGAGCCAC	65	443	376	201
	GAGTGGACAGATCCCCAAAGGACTCAAGGA	65		67	175
$\beta^E$ : CD 26 (G→A) (Loss of <i>MnII</i> site)	ACCTCACCCCTGTGGAGCCAC	65	443	231	171
	GAGTGGACAGATCCCCAAAGGACTCAAGGA			89/56/35/33	89/60/35/33
$\beta^D$ Punjab: CD 121 (G→C) (Loss of <i>EcoRI</i> site)	CAATGTATCATGCCTCTTTGCACC	65	861	861	552
	GAGTCAAGGCTGAGAGATGCAGGA	65			309
$\beta^O$ Arab: CD 121 (G→A) (Loss of <i>EcoRI</i> site)	CAATGTATCATGCCTCTTTGCACC	65	861	861	552
	GAGTCAAGGCTGAGAGATGCAGGA	65			309



2. Both positive and negative DNA controls should always be tested alongside any sample. For prenatal diagnosis this usually means a normal, heterozygous and homozygous DNA sample for the mutation being studied.
3. The relationship between fragment intensities after staining should be constant for all DNA samples. Any deviation to the expected pattern of band intensities in a particular sample should be treated as suspect and the sample should be retested (for example, as a result of poor amplification of one of the two alleles, or a partial digestion of the amplified product by the restriction enzyme).
4. Sometime an ARMS primer may produce a faint positive response with its negative DNA control. This is usually less intense than that of the product observed in the positive DNA control, and may be a false-positive result. This occurs if there has been a subtle change in the reaction conditions resulting from less than optimal performance of the heating block or if the ARMS primer has started to lose its specificity through degradation. Always use small aliquots of primers as a working solution, and store the stock solution at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$  if available.
5. Failure of the PCR to produce any product may be the result of the genomic DNA sample not reaching the recommended concentration of  $100\text{ ng}/\mu\text{L}$ , especially if the sample has been referred from another laboratory. Quantify the DNA sample and if it is too dilute, reprecipitate in a smaller volume. More often, the DNA is too concentrated, and a 1-in-10 dilution can be attempted. Do not increase the number of cycles to obtain a result, as nonspecific amplification products are likely to appear and there may be a problem of co-amplifying any contaminating maternal DNA in a fetal sample. Amplification failure may also be caused by an error in the primer sequence. Check the published sequence against the GenBank sequence for typing errors!
6. Finally, an unexpected failure of all the PCR tests set up in one batch usually results from a problem with the dNTP mixture. The first troubleshooting step should be to try new dNTP solutions.

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## Cystic Fibrosis

Harry Cuppens, Elisabeth Dequeker, and Jean-Jacques Cassiman

### 1. Introduction

#### 1.1. *Diagnostics of Cystic Fibrosis*

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (1,2). More than 1000 disease mutations and 200 polymorphisms have been identified in the *CFTR* gene (3). The type and frequency of mutations is very variable from ethnic population to population (4). The most common mutation, F508del (e.g.,  $\Delta F508$  according to the old nomenclature), reaches frequencies of about 70% in Northern European populations. Besides F508del, other common mutations exist in most populations, each reaching frequencies of approx 1–2%. Examples include the 1717-1G→A, G542X, G551D, R553X, W1282X, and N1303K mutations. Finally, for a given ethnic population, “ethnic-specific” mutation(s) that reach frequencies of more than 1–2% might exist. For most ethnic populations, “common” mutations cover about 85% of all mutant *CFTR* genes. The remaining group of mutant *CFTR* genes in a particular ethnic population comprises rare mutations, some of them only found in a single family. Moreover, when one of the more common mutations is not found in a patient, a mutation is likely to be present that has never been detected before in that ethnic population. It is therefore very difficult to set up a strategy for a general screening of *CFTR* mutations, which allow sensitivities close to 100%, even in a well-characterized ethnic population.

*CFTR* mutation screening, like other genetic tests, is complex and prone to errors. Although in yearly European quality-control studies, a decrease in error rate is noted (5), the error still remains unacceptably high. In the quality-control study of 2000, in which 191 laboratories participated, 19 (10%) of the

labs were not able to type all the DNA samples studied correctly. Testing and screening for *CFTR* mutations should therefore be performed according to consensus quality assurance guidelines, which have been described elsewhere (4,6). For each *CFTR* mutation, different approaches might exist for mutation analysis, depending on the familiarity and expertise with specific assays in a given laboratory. In a survey of 151 European laboratories (5), five tests are most frequently used: restriction enzyme analysis, the heteroduplex assay, the Elucigene™ CF assay, the INNO-LiPA *CFTR* assay, and the OLA CF assay. Tests based on restriction enzyme analysis are straightforward, once the identity of a mutation is known, and are not discussed further here. The four other assays are described in more detail here.

The heteroduplex assay is a very quick assay for the detection of the most common mutation F508del, and is thus well-suited for the initial screening for *CFTR* mutations. The Elucigene™ CF, the INNO-LiPA *CFTR*, and the OLA CF assays are popular because they allow the simultaneous detection of up to 31 *CFTR* mutations thus far. Mutations detected by the commercial tests have been selected on the basis of their reported prevalence in the Caucasian population. Also included in these assays are the most frequent ethnic-specific mutations. If in a particular ethnic population one of the latter tests is used, and screening for additional mutations is still needed, specific tests should then be set up by the laboratory. The systematic testing for well-defined mutations that constitute the group of rare mutations (about 15%) may not be practical and economical. Moreover, a mutant *CFTR* gene might harbor a mutation type that has never been detected before in that given population, and the screening of defined mutations may therefore be unsuccessful. Therefore, a screening protocol for the presence of an unknown mutation in the coding region and exon/intron junctions of the *CFTR* gene may be performed. Screening systems include single-strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), and sequencing. DGGE, DHPLC, and sequencing protocols specific for the *CFTR* gene have been described elsewhere (7–9). Mutations in intronic regions and the promoter region may still be missed, and would need to be screened at the *CFTR* transcript level. However, this may be difficult in current routine diagnostic settings.

## 1.2. *CFTR*-Related Diseases

Apart from its involvement in CF, *CFTR* is also involved in other diseases such as congenital bilateral absence of the vas deferens (CBAVD) (10,11), disseminated bronchiectasis (12), and chronic pancreatitis (13). *CFTR* tests can therefore be also used for diagnostic purposes for these diseases. It should be noted that two mutations will not always be found on both *CFTR* genes in these

patients. Other genetic factors are believed to be involved in patients who do not harbor *CFTR* mutations on both *CFTR* genes. In patients having two mutant *CFTR* genes, at least one will be a “mild” mutation. It should be noted that the majority of mutations for which one screens in commercial tests are severe mutations, so that mild mutations may not be detected. The most frequent mutation that confers a mild phenotype found in these patients is the T5 polymorphism at the polymorphic intron 8 Tn locus (**14**). The Elucigene™ CF assay and the INNO-LiPA *CFTR* assay allow genotyping at the Tn locus. It should be noted that T5 is a disease mutation with partial penetrance. The penetrance can be explained in part by additional loci, such as the (TG)<sub>m</sub> locus upstream of the Tn locus (**15**). If the R117H mutation is found, the results at the Tn locus should be interpreted in combination with this mutation, since R117H can be either found in combination with a T5 or T7 allele. R117H-T5 is only found in CF patients, and R117H-T7 is found both in CF and CBAVD patients (**16**).

### **1.3. The Heteroduplex Assay**

Heteroduplexes from polymerase chain reaction (PCR) products, derived from genomic DNA of an individual, can be formed when a deletion (insertion) is found in at least one of the two different PCR product populations. Moreover, the two heteroduplex populations (sense mutant/anti-sense wild-type and sense wild-type/anti-sense mutant) might also have different electrophoretic mobilities. The presence of heteroduplexes in the PCR products shows that the individual from whom the sample is derived is heterozygous for the mutation. In individuals who are homozygous for the deletion/insertion or wild-type allele, the genotype can be determined based on these heteroduplexes. In this situation, mixing of the PCR sample with PCR products of a reference sample (for example, PCR products derived from an individual homozygous for the wild-type allele), will allow the formation of heteroduplexes. If heteroduplexes are found, the individual will be homozygous for the allele that is not present in the reference sample. If no heteroduplexes are found, the individual will be homozygous for the same allele that is found in the reference sample. Based on this principle, the most common mutation, F508del, can be screened in an easy and quick heteroduplex assay of exon 10 of the *CFTR* gene. This assay is then also commonly used as a first initial screening for a *CFTR* aberration in patients.

### **1.4. The Elucigene™ CF Assay**

This assay is based on the Amplification Refractory Mutation System (ARMS™) technology, which can detect point mutations or small DNA deletions (**Fig. 1**). Selection of the appropriate primers allows specific mutant or normal DNA sequences to be amplified and detected. Detection of the

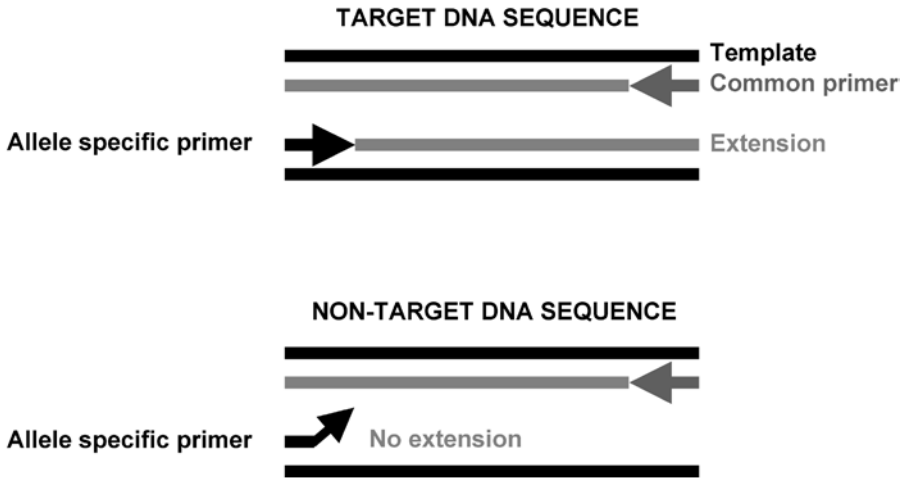


Fig. 1. Principle of the Elucigene™ CF assay.

products generated by the procedure is performed by gel electrophoresis, staining, and subsequent viewing under ultraviolet (UV) transillumination. Comparison of the PCR product position in the gel against an adjacent marker track allows identification of the mutation. The Elucigene™ CF 20 assay allows the detection of 20 mutations (**Table 1**). Except for the F508del mutation, this test does not distinguish between the homozygous or heterozygous state for these mutations. This assay also allows genotyping at the Tn locus in intron 8 of the *CFTR* gene.

### 1.5. The INNO-LiPA *CFTR* Assay

INNO-LiPA *CFTR* tests are based on the reverse hybridization principle (**Fig. 2**). Specific oligonucleotide probes that detect different alleles in the *CFTR* gene are immobilized as parallel lines on membrane strips. Amplified biotinylated DNA, obtained in a multiplex PCR reaction, is chemically denatured, and the single DNA strands are hybridized with these oligonucleotide probes. After hybridization and stringent washing, streptavidin labeled with alkaline phosphatase is added, and will bind to the biotinylated hybrids. The membranes are then incubated with a substrate solution so that purple/brown precipitates are formed at the position of the hybrids. The INNO-LiPA *CFTR*12 assay allows the detection of 12 mutations, the INNO-LiPA *CFTR*17 + Tn assay allows the detection of 17 mutations and genotyping of the Tn locus in intron 8 of the *CFTR* gene (**Table 1**). All mutations can be typed in the homozygous and heterozygous state.



**Table 1**  
**Mutations That Can Be Detected with Different *CFTR* DNA Tests**

The Elucigene™ CF assay

E60X, R117H, 621+1G→T, 1078delT, R334W, R347P, A455E, I507del, F508del, 1717-1G→A, G542X, G551D, R553X, 2183AA→G, R1162X, 3659delC, 3849+10kbC→T, S1251N, W1282X, N1303K, Tn

The INNO-LiPA *CFTR* assay

*INNO-LiPA CFTR12*:

I507del, F508del, 1717-1G→A, G542X, G551D, Q552X, R553X, R560T, S1251N, 3905insT, W1282X, and N1303K

*INNO-LiPA CFTR17+Tn*:

E60X, G85E, 394delTT, R117H, 621+1G→T, 711+5G→A, 1078delT, R334W, R347P, A455E, 2143delT, 2183AA→G, 2184delA, 2789+5G→A, R1162X, 3659delC, 3849+10kbC→T, Tn

The OLA CF assay

G85E, R117H, Y122X, 621+1G→T, 711+1G→T, 1078delT, R334W, R347H, R347P, A455E, I507del, F508del, 1717-1G→A, Q493X, V520F, G542X, S549N, S549R, G551D, R553X, R560T, 1898+1G→A, 2183AA→G, 2789+5G→A, R1162X, 3659delC, 3849+4A→G, 3849+10kbC→T, 3905insT, W1282X, and N1303K

### 1.6. The OLA Cystic Fibrosis Assay

In the OLA (oligonucleotide ligation assay) CF test, domains of the *CFTR* gene in which the mutations to be tested are located, are first amplified in a multiplex amplification, followed by an OLA amplification (**Fig. 3**). In this reaction, three oligonucleotide primers are required for each amplified *CFTR* domain (amplicon). A common primer hybridizes at a sequence common to both the mutant and normal *CFTR* alleles. The two allelic primers—the mutant primer and the wild-type primer—compete for binding to the PCR amplicon. The primer that is the perfect complement to the *CFTR* amplicon will hybridize to the DNA and will be ligated to the common primer, and an OLA product is formed. Each OLA product can be identified after electrophoresis based on its electrophoretic mobility and fluorescence. If no mutations are present at a specific *CFTR* locus, the wild-type primer and the common primer will bind to that allele. If a homozygous mutation is present at a specific *CFTR* locus, the mutant and the common primer will bind to the DNA. When the DNA sample is heterozygous for that mutation, the wild-type and mutant allelic primers and the common probe will bind at the respective normal and mutant loci. The OLA

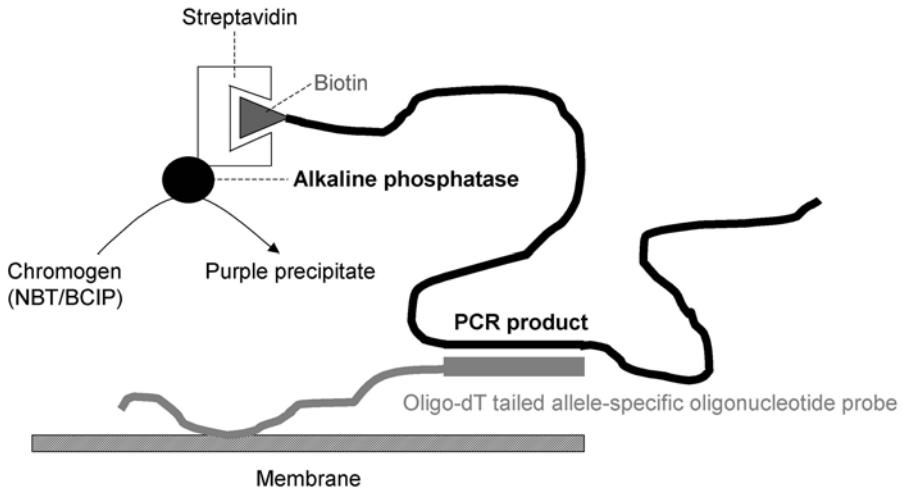


Fig. 2. Principle of the INNO-LiPA *CFTR* assay.

CF assay allows the detection of 31 mutations in homozygous and heterozygous state (Table 1).

### 1.7. Sensitivity of Assays

The number of mutations that can be simultaneously detected in currently available tests is limited, but they provide extensive mutation detection coverage across most European populations. Also, relatively common ethnic-specific mutations are included. However, it should be noted that the first mutations to be historically identified have been thoroughly screened in most populations, yet this is not the case for mutations that were identified later. Therefore, the frequency of some mutations may be still underestimated. The commercial tests described here have recently been updated—e.g., development of a new generation assay that allows detection of more mutations. Newer generations may continue to be developed. One way to increase the number of mutations that can be screened simultaneously is the exclusion of wild-type probes of each mutant locus. This strategy has been used in the Elucigene™ CF20 assay. Here, only the wild-type allele for the F508 position has been included, yet for the remaining 19 mutations the probe for the wild-type allele was not included. The reasoning is that the number of homozygotes for these 19 mutations is very small (less than 0.25%). Wild-type probes for these mutant loci were therefore not included in this assay, since it would only result in a modest increase of the sensitivity of the assay. For a limited number of oligonucleotides that can be included in the assay (e.g., 20), a higher sensitivity will be obtained when only the mutant probes are included (20 mutations) than when both mutant and

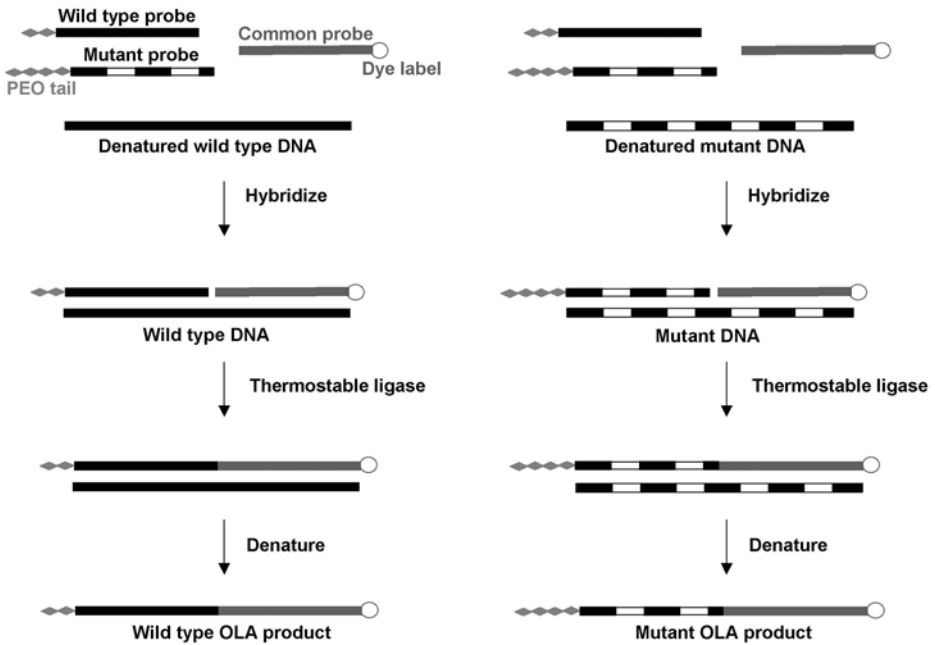


Fig. 3. Principle of the OLA CF assay.

wild-type probes are included (10 mutations). However, exclusion of the wild-type probes results in a lower quality of genotype information (e.g., not having a definite knowledge of the homozygous or heterozygous presence of a particular mutation). The heterozygous or homozygous state, especially for mutations of intermediate frequency (1717-1G→A, G542X, G551D, R553X, W1282X, and N1303K), should then be confirmed with an alternative assay. Another way to determine the heterozygous or homozygous state of a mutation is to screen *CFTR* mutations in the parents of the proband. In the INNO-LiPA *CFTR* and OLA CF assays, wild-type oligonucleotide probes for each locus have been included. Apart from allowing discrimination between the heterozygous or homozygous state of a particular mutation, the inclusion of wild-type oligonucleotide probes also provides an internal control for successful amplification of each of the fragments of the complex multiplex reaction. However, even when wild-type oligonucleotides are included, the homozygous observation of a rare mutation is not error-proof. There are many variant nucleotides in the *CFTR* gene that are not detected in these tests, but some of them may cause null alleles by interfering with the hybridization of oligonucleotides. For the more frequent mutations/polymorphisms, one is able to optimize the specificity of allele-specific oligonucleotides (ASOs) that also contain the nucleotide affected by such a mutation, so that the correct genotype is determined. However, it is

impossible to optimize the test for all rare mutations or new mutations that have not yet been identified. During quality-control studies, it was found that compound heterozygosity for mutations, in which the affected nucleotide of one mutation is located in an ASO probe for another mutation, is a frequent cause for an error in genotyping (5), even if these problems have been addressed in the manufacturer's information (e.g., diagnosis of a compound heterozygote for the G551D and R553X mutations). This problem may be encountered with any oligonucleotide, whether it is a PCR primer, wild-type, or mutant ASO probe, in any screening assay. Deletions that have given rise to apparent homozygous mutants in these PCR-based assays could be another error—e.g., a compound heterozygote for F508del and an exon 10 deletion could be typed as a F508del homozygote. Whenever an aberrant pattern is obtained, the sample should be re-examined, possibly by an alternative method. Screening of the parents (paternity proven) may resolve the issue.

## 2. Materials

### 2.1. The Heteroduplex Assay

1. DNA thermal cycler.
2. Electrophoresis equipment (vertical electrophoresis chamber, gel plates, spacers and combs, power supply).
3. Taq DNA polymerase (5 U/ $\mu$ L).
4. Mineral oil (optional).
5. 10  $\mu$ M primer *CFTR*.ex10i-5 (5'-GCA GAG TAC CTG AAA CAG GA-3').
6. 10  $\mu$ M primer *CFTR*.ex10i-3 (5'-CAT TCA CAG TAG CTT ACC CA-3').
7. 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM MgCl<sub>2</sub>, 0.1% gelatin).
8. 2 mM deoxynucleotide 5' triphosphate (dNTP) mix: 2 mM deoxyadenosine triphosphate (dATP), 2 mM deoxycytidine triphosphate (dCTP), 2 mM deoxyguanosine triphosphate (dGTP), 2 mM deoxythymidine triphosphate (dTTP).
9. TE buffer pH 8.00 (1 mM Tris-HCl, 0.1 mM EDTA).
10. 10X TBE buffer (900 mM Tris-borate, 20 mM EDTA).
11. 6X loading buffer (0.25% bromophenol blue, 0.125% xylene cyanol, 40% sucrose, 0.2 M EDTA).
12. 6% polyacrylamide gel in 1X TBE (acrylamide-bisacrylamide:37.5/1).
13. 0.5  $\mu$ g/mL ethidium bromide.
14. DNA ladder.

### 2.2. The Elucigene™ CF Assay

1. DNA thermal cycler.
2. Electrophoresis equipment (horizontal electrophoresis chamber, gel tray, comb, power supply).

3. Mineral oil (optional).
4. TE buffer pH 8.00 (1 mM Tris-HCl, 0.1 mM EDTA).
5. 10X TBE (Elucigene) buffer (1.34 M Tris-base, 0.749 M boric acid, 25.5 mM EDTA).
6. NuSieve 3:1 Agarose (BioWhittaker Molecular Applications, Rockland, ME), in 1X TBE (Elucigene).
7. Ethidium bromide.
8. 50-bp ladder (Amersham Pharmacia Biotech, Uppsala, Sweden).
9. Elucigene™ CF20 and/or Elucigene™ CF Poly-T kit (Cellmark Diagnostics, Oxfordshire, UK).

### **2.3. The INNO-LiPA CFTR Assay**

1. DNA thermal cycler.
2. *Auto*-LiPA apparatus (Innogenetics NV, Ghent, Belgium) (optional).
3. Water bath with shaking platform (80 rpm; with inclined lid; temperature adjustable to 47°C ± 0.5°C).
4. Aspiration apparatus.
5. Calibrated thermometer.
6. Orbital, reciprocal, or rocking platform shaker.
7. Forceps for strip-handling.
8. Taq DNA polymerase (5 U/μL).
9. Mineral oil (optional).
10. Distilled or deionized water.
11. TE buffer pH 8.00 (1 mM Tris-HCl, 0.1 mM EDTA).
12. INNO-LiPA *CFTR*12 amplification reagents and/or INNO-LiPA *CFTR*17 + Tn amplification reagents.
13. INNO-LiPA *CFTR*12 and/or INNO-LiPA *CFTR*17+ Tn kit (Innogenetics NV).

### **2.4. The OLA CF Assay**

1. DNA thermal cycler (preferentially GeneAmp® PCR system 9700, 9600, or 2400 (Applied Biosystems [ABI], Foster City, CA).
2. ABI PRISM® 310 Genetic Analyzer, ABI PRISM® 373 DNA Sequencer, or ABI PRISM® 377 DNA Sequencer (ABI).
3. GeneScan® Analysis Software (ABI).
4. Genotyper® Software (ABI).
5. CF Genotyper Template (ABI).
6. TE buffer pH 8.00 (1 mM Tris-HCl, 0.1 mM EDTA).
7. OLA CF assay (ABI).

## **3. Methods**

### **3.1. The Exon 10 Heteroduplex Assay**

#### **3.1.1. Amplification**

1. Dilute each genomic DNA sample to a concentration between 100 and 500 ng/μL in TE buffer (**Note 1**).

- Determine the amount of solution to be prepared based on number of vials (N) that are needed ( $n = \text{number of DNA samples} + 1$  [negative control; no DNA] + 1).

Using cotton-plugged pipet tips, prepare a master mix in an autoclaved 1.5-mL tube:

- ( $N \times 36.75 \mu\text{L}$ ) autoclaved distilled water:
- + ( $N \times 5 \mu\text{L}$ ) 10X PCR buffer.
- + ( $N \times 5 \mu\text{L}$ ) 2 mM dNTP mix.
- + ( $N \times 1 \mu\text{L}$ ) primer *CFTR.ex10i-5* (10  $\mu\text{M}$ ) (**Note 2**).
- + ( $N \times 1 \mu\text{L}$ ) primer *CFTR.ex10i-3* (10  $\mu\text{M}$ ) (**Note 2**).
- + ( $N \times 0.25 \mu\text{L}$ ) Taq DNA polymerase (5 U/ $\mu\text{L}$ )

Vortex briefly and aliquot 49  $\mu\text{L}$  of this master mix into (N-1) autoclaved amplification tubes.

- Add one drop of mineral oil into each tube if required.
- Pipet 1  $\mu\text{L}$  (100–500 ng) of the genomic DNA (through the mineral oil if required).
- Add 1  $\mu\text{L}$  of distilled water (no DNA) to the negative control tube.
- Perform a PCR with amplification profile; initial denaturation for 5 min at 94°C; denaturation for 1 min at 94°C, annealing for 1 min at 62°C, extension for 1 min at 68°C, for a total of 30 cycles; and a final extension step for 10 min at 68°C.

### 3.1.2. Heteroduplex Analysis

- Mix 5  $\mu\text{L}$  PCR products of each sample with 5  $\mu\text{L}$  PCR products derived from a known non-carrier. A control could be an assay in which 5  $\mu\text{L}$  PCR products of each sample is mixed with 5  $\mu\text{L}$  PCR products derived from a known F508del/F508del CF patient.
- Denature for 7 min at 94°C, renature for 15 min at 42°C, and finally let cool to room temperature.
- For each sample, add 2  $\mu\text{L}$  6X loading buffer to 10  $\mu\text{L}$  untreated PCR product and 2  $\mu\text{L}$  6X loading buffer to 10  $\mu\text{L}$  sample of the heteroduplex assays.
- Perform the electrophoresis on a 6% polyacrylamide gel in 1X TBE.
- Stain the gel in ethidiumbromide solution.
- View the gel under UV transillumination, and make a photograph.

### 3.1.3. Evaluation

Some examples are shown in **Fig. 4**. The wild-type homoduplexes have a length of 491 bp. If heteroduplexes are already found in the PCR sample alone, as well as in the samples in which the heteroduplex formation assays were performed, the patient is heterozygous for a deletion mutation. It should be noted that F508del/wild-type allele heteroduplexes have a characteristic electrophoretic mobility that differs from I507del/wild-type allele heteroduplexes. The same may be true for other rarer deletions/insertions, as shown in **Fig. 4**. If the individual is homozygous for the wild-type allele, only heteroduplexes will be found in the sample in which PCR products of the known

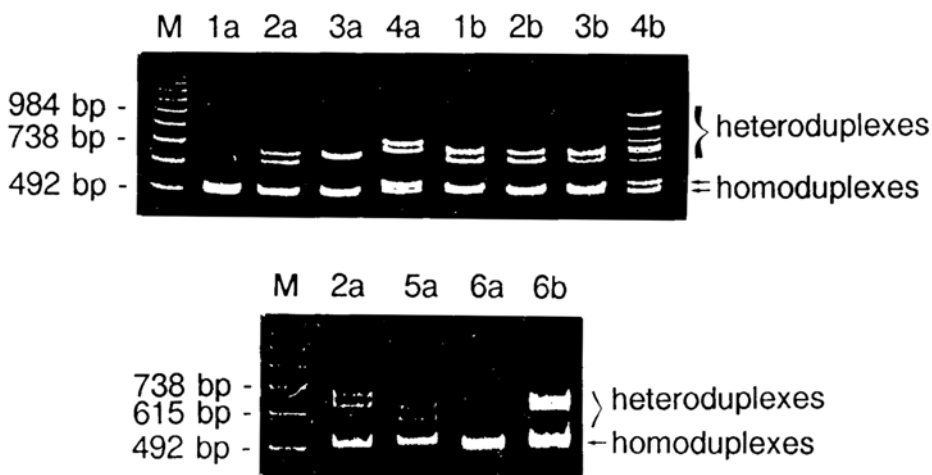


Fig. 4. Amplification and heteroduplex formation of different *CFTR* mutations. Lane 1a shows the amplification products of a homozygous normal individual; lane 2a, from a heterozygous F508del individual; lane 3a, from a heterozygous I507del individual; lane 4a, from a heterozygous dup1716+(51→61) individual; lane 5a, from a heterozygous 1609delCA individual; and lane 6a, from a homozygous 609delCA individual. In lanes (b), the same samples are shown; however, they were denatured and reannealed after the addition of PCR products of a homozygous F508del individual. M denotes the 123-bp ladder.

F508del/F508del CF patient were added, no heteroduplexes will be found in the sample in which PCR products of a known non-carrier were added. If the individual is homozygous for the F508del mutation, only heteroduplexes will be found in the sample in which PCR products of the known non-carrier were added, and no heteroduplexes will be found in the sample in which PCR products of the known F508del/F508del CF patient were added. If two different deletion/insertion mutations are present in the sample, a more complex pattern of heteroduplexes will be found after PCR and/or the heteroduplex assay, as shown in **Fig. 4** (F508del/dup1716 + (51→61)/wild-type). Given the characteristic electrophoretic mobility of the more common F508del/wild-type allele and I507del/wild-type allele heteroduplexes, any unfamiliar heteroduplex pattern requires further investigation and is most likely indicative of the presence of a different mutation in exon 10 on at least one of the mutant *CFTR* genes.

### 3.2. The Elucigene<sup>®</sup> CF Assay

#### 3.2.1. Amplification

1. Dilute each genomic DNA sample to a concentration between 2 and 20 ng/ $\mu$ L in TE buffer (**Note 1**).

2. a. For the Elucigene™ CF 20 kit:  
Determine the amount of solution to be prepared based on number of vials (N) that are needed ( $3 \times n$  (number of DNA samples) + 1 (negative control; no DNA) + 1). Using cotton-plugged pipet tips, prepare enzyme mix in an autoclaved 1.5-mL tube:  
( $N \times 6.8 \mu\text{L}$ ) autoclaved distilled water.  
+ ( $N \times 2 \mu\text{L}$ ) dilution buffer.  
+ ( $N \times 10 \mu\text{L}$ ) loading dye (allows a visual check that the dilution has been adequately mixed and that the dilution will be subsequently added to vials at a later stage).  
+ ( $N \times 1.2 \mu\text{L}$ ) AmpliTaq Gold.  
Vortex briefly.
- b. For the Elucigene™ CFpoly-T kit:  
Determine the amount of solution to be prepared based on number of vials (N) that are needed ( $n$  (number of DNA samples) + 1 (negative control; no DNA) + 1). Using cotton-plugged pipet tips, prepare enzyme mix in an autoclaved 1.5-mL tube:  
( $N \times 1.7 \mu\text{L}$ ) autoclaved distilled water.  
+ ( $N \times 0.5 \mu\text{L}$ ) dilution buffer.  
+ ( $N \times 2.5 \mu\text{L}$ ) loading dye.  
+ ( $N \times 0.3 \mu\text{L}$ ) AmpliTaq Gold.  
Vortex briefly.
3. Defrost  $n + 1$  (for  $n$  DNA samples and one extra vial for the negative control) vials A, B, and C (and, if tested, the vial containing amplification solution for the poly-T alleles) and centrifuge for 10 s at 12,000g. Each of the vials A, B and C contains a set of mutations (**Fig. 5**).
4. Add 5  $\mu\text{L}$  enzyme mix to each vial, using separate cotton-plugged pipet tips.
5. Add 5  $\mu\text{L}$  of each DNA sample to be tested into each of a vial A, B, and C trio (and, if tested, in the poly-T vial).
6. For the negative control, add 5  $\mu\text{L}$  distilled water to a vial A, B, and C trio (and, if tested, in the poly-T vial).
7. Add one drop of mineral oil into each tube if required.
8. Centrifuge all the amplification vials until all liquid is at the bottom of each vial.
9. Perform the PCR using the following amplification profile: initial denaturation for 20 min at 94°C; denaturation for 30 s at 94°C, annealing for 2 min at 58°C, extension for 1 min at 72°C, for a total of 35 cycles; and a final extension step for 20 min at 72°C. Upon completion, the samples may be stored at room temperature overnight or at 2–8°C for up to 7 d before analysis by gel electrophoresis.

### 3.2.2. Electrophoresis

1. Results can be obtained in 15  $\times$  12 cm horizontal submarine gels with combs of 1.5 mm  $\times$  5 mm suspended 1 mm above the gel tray, using 100 mL of 3% NuSieve 3:1 agarose in 1X TBE (Elucigene) buffer with 0.1  $\mu\text{g}/\text{mL}$  ethidiumbromide.



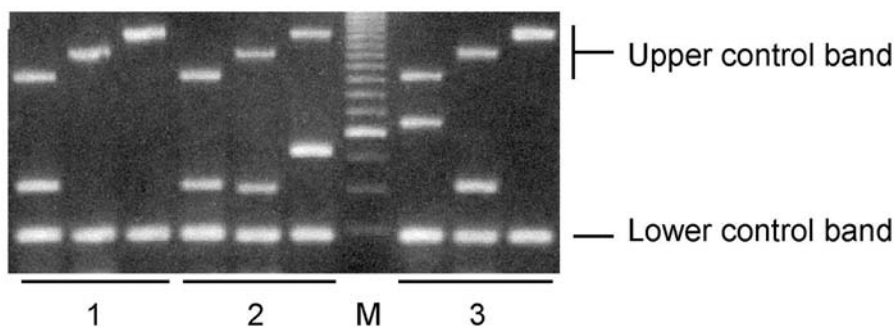


Fig. 5. Representative illustration of results obtained with the Elucigene™ CF20 assay. Sample 1 is derived from a homozygous F508del individual; sample 2 is derived from a compound heterozygous F508del/I507del individual; and sample 3 is derived from a heterozygous G542X individual. M denotes the mol-wt marker. The length of each fragment that is obtained corresponds to a particular allele: Reaction A: upper control ApoB:423 bp, 1717-1G→A:329 bp, G542X:279 bp, W1282X:240 bp, N1303K:200 bp, F508del:160 bp, 3849 + 10kbCT:132 bp, lower control ODC:97 bp; Reaction B: upper control ApoB:526 bp, 621 + 1G→T:383 bp, R553X:328 bp, G551D:290 bp, R117H:243 bp, R1162X:200 bp, F508 (wild-type):160 bp, R334W:140 bp, lower control ODC:97 bp; Reaction C: upper control ApoB:633 bp, A455E:500 bp, 2183AA→G:425 bp, 3659delC:344 bp, 1078delT:272 bp, I507del:222 bp, R347P:184 bp, S1251N:150 bp, E60X:121 bp, lower control ODC:97 bp; Poly-T reaction: 9T:169 bp, 7T:132 bp, 5T:105 bp.

1X TBE (Elucigene) buffer, containing 0.1 µg/mL ethidiumbromide is used as running buffer.

2. Prepare a diluted 50-bp ladder solution, using a 10-µL 50-bp ladder (1.5 µg/15 µL), 80 µL distilled water, and 10 µL loading dye. 15 µL of diluted 50-bp ladder solution should be loaded on the gel and run adjacent to the samples.
3. 15 µL of the PCR product (with the loading dye added during the PCR set-up process) should be loaded on the gel. Each A, B, and C vial trio is loaded into adjacent wells. If tested, 15 µL of PCR products of the poly-T vial is also loaded. Preferentially all poly-T samples are loaded in adjacent wells.
4. Perform the electrophoresis at 5–6 V/cm between electrodes until the dye front has migrated 5 cm from the loading wells toward the anode (1.5–2 h).
5. After electrophoresis, place the gel on an UV transilluminator at 260 nm to visualize the bands and photograph.

### 3.2.3. Evaluation

Representative examples are shown in **Fig. 5**. The length of each fragment that is obtained corresponds to a particular allele. For the A, B, and C reactions, as confirmation of amplification, all controls in the three multiplexes must be

positive (fragment of the human Apolipoprotein B gene [upper control] and fragment of the ornithine decarboxylase gene [lower control]). Except for the F508 position, no primers are included that detect the normal alleles for the other 19 mutant loci. The negative control should contain no amplification products. For the poly-T reaction, as confirmation of amplification, at least one diagnostic band should be seen. Because of the polymorphic nature of the poly-T locus, as well as the presence of a polymorphic TG repeat just upstream of the poly-T locus, heteroduplexes might be formed. However, because of their faint signal and their electrophoretic mobility, they still allow a correct interpretation of the diagnostic bands. If a band is seen in the mutant F508del position only, the patient is homozygous for the F508del mutation, if only a band is seen for the wild-type allele at position F508 the patient does not carry F508del on either *CFTR* gene, if a band is seen both for the mutant F508del and for the wild-type allele at position F508, the patient is (compound) heterozygote for the F508del mutation. As long as two mutations are positive with the assay, a compound heterozygous genotype for these two mutations can be established. Except for the F508del mutation, if only one mutation is found, the patient is either heterozygous (true heterozygous or compound heterozygote for this mutation and a second mutation not detected with the assay) or homozygous for this mutation. For the poly-T reaction, if only one band (5T, 7T, or 9T) is seen, the patient will be homozygous for the respective allele. If two bands are seen, the patient will be compound heterozygote for these two respective alleles. The mutations R117C, R117L, 621 + 2T→C, I506V, I507V, F508C, 1717-2A→G, R1162L, R1283M have been shown to not result in crossreactivity. However, R347H crossreacts with R347P, resulting in a faint PCR product visible at the R347P position in reaction C. 2184insG will theoretically crossreact with the 2183AA→G primer in reaction C. Whenever an abnormal pattern is obtained, the sample should be re-examined, and an alternative method will be needed.

### 3.3. The INNO-LiPA *CFTR* Assay

#### 3.3.1. Amplification

1. Dilute each genomic DNA sample to a concentration between 0.05 and 0.3 µg/µL in TE buffer (**Note 1**).
2. Determine the amount of solution to be prepared based on number of vials (N) that are needed ( $n = \text{number of DNA samples} + 1$  (negative control; no DNA) + 1). One amplification (PS1) should be performed when the INNO-LiPA *CFTR*12 assay is used, two separate amplifications (PS2 and PS3) should be performed when the INNO-LiPA *CFTR*17 + Tn assay is used. Perform the pipetting steps on ice and without delay. Using a cotton-plugged pipet tip, prepare a master mix in an autoclaved 1.5-mL tube:

( $N \times 24.7 \mu\text{L}$ ) autoclaved distilled water.

+ ( $N \times 10 \mu\text{L}$ ) amplification buffer (AB).

+ ( $N \times 10 \mu\text{L}$ ) primer solution PS1 for *CFTR*12, or primer solution PS2 for *CFTR*17 + Tn, or primer solution PS3 for *CFTR*17 + Tn.

+ ( $N \times 0.3 \mu\text{L}$ ) Taq DNA polymerase ( $N \times 1.5 \text{ U}$ ) ( $5 \text{ U}/\mu\text{L}$ ).

The total volume of this amplification mix is now ( $N \times 45 \mu\text{L}$ ). Vortex briefly and aliquot  $45 \mu\text{L}$  of a master mix into ( $N-1$ ) autoclaved amplification tubes.

3. Add two drops of mineral oil into each tube if required.
4. Pipet  $5 \mu\text{L}$  ( $0.25\text{--}1.5 \mu\text{g}$ ) of the genomic DNA (through the mineral oil if required).
5. Add  $5 \mu\text{L}$  of distilled water (no DNA) to the negative control tube.
6. Place the samples into the preheated and calibrated thermal block ( $95^\circ\text{C}$ ) and perform the PCR using the following amplification profile: initial denaturation for 5 min at  $95^\circ\text{C}$ ; denaturation for 1 min at  $95^\circ\text{C}$ , annealing for 1 min at  $57^\circ\text{C}$ , extension for 1 min at  $68^\circ\text{C}$ , for a total of 30 cycles; and a final extension step for 10 min at  $68^\circ\text{C}$ .
7. After the amplification process, use the samples immediately with the INNO-LiPA strips or store the samples at  $-15^\circ\text{C}$  to  $-25^\circ\text{C}$ . (Optional: The presence of amplified products can be verified by electrophoresis, subsequent staining with ethidium bromide, and visualization under UV transillumination.)

### 3.3.2. Denaturation and Hybridization (**Note 3**)

1. Heat a shaking water bath to  $47^\circ\text{C} \pm 0.5^\circ\text{C}$ . Check the temperature using a calibrated thermometer. Do not exceed the indicated temperature. Prewarm the Hybridization Solution (HS) and Stringent Wash Solution (SW) in a water bath of at least  $37^\circ\text{C}$ , and do not exceed  $47^\circ\text{C}$ . Mix before use. All crystals should be dissolved.
2. Using forceps, remove the required number of INNO-LiPA *CFTR* strips from the tube (one strip per test sample). Include a strip for the blank amplified control sample. Pencil an identification number above the black marker line on the strip (do not touch the strips with bare hands).
3. Take the required number of test troughs (one trough per test sample) and place them into the tray.
4. For INNO-LiPA *CFTR*12, pipet  $10 \mu\text{L}$  Denaturation Solution (DS) into the upper corner of each trough (close the vial immediately after use); for INNO-LiPA *CFTR*17 + Tn, pipet  $20 \mu\text{L}$  Denaturation Solution (DS).
5. Add  $10 \mu\text{L}$  PS1-amplified product for INNO-LiPA *CFTR*12; for INNO-LiPA *CFTR*17 + Tn, add  $10 \mu\text{L}$  PS2- and  $10 \mu\text{L}$  PS3-amplified products; and carefully mix by pipetting up and down. Always use sterile pipet tips. Allow denaturation to proceed for 5 min at  $20\text{--}25^\circ\text{C}$ .
6. Shake the prewarmed Hybridization Solution (HS) and gently add 2 mL to the denatured amplified product in each trough. Mix by gentle shaking. Take care not to contaminate neighboring troughs during pipetting.
7. Immediately place the strip with the marked side of the membrane upward into the trough. The strips should be completely submerged in solution (wear disposable gloves and use forceps).

8. Place the tray into the 47°C shaking water bath (80 rpm), close the lid, and incubate for 90 min (avoid splashing water from the water bath into the trough; adjust the water level between 1/3 and 1/2 of the height of the trough; if necessary, prevent the tray from sliding by immobilizing the tray between two heavy weights).

### 3.3.3. Stringent Wash (**Note 3**)

1. After hybridization, remove the tray from the water bath.
2. Hold the tray at a low angle and aspirate the liquid from the trough with a pipet, preferably attached to a vacuum aspirator. Add 2 mL prewarmed Stringent Wash solution (SW) into each trough and rinse by rocking the tray briefly (10–20 s) at 20–25°C. Aspirate the solution from each trough. Repeat this brief washing step once.
3. Aspirate solution and incubate each strip in 2 mL prewarmed Stringent Wash solution (SW) in the shaking water bath at 47°C ± 0.5°C for 30 min. Always close the lid of the water bath (before incubation, check the temperature of the water bath using a calibrated thermometer, and adjust the temperature if necessary).

### 3.3.4. Color Development (**Note 3**)

All subsequent incubations are carried out at 20–25°C on a shaker. During the incubations, the liquid and test strips should move longitudinally back and forth in the trough for homogeneous staining.

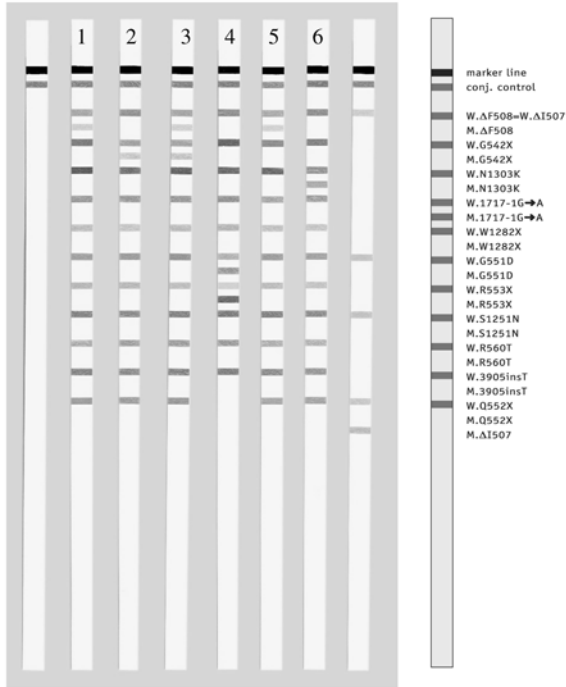
1. Prepare Rinse solution by diluting concentrated Rinse Solution (RS) 1:5 (1 part + 4 parts) in distilled or deionized water. Prepare 8 mL Rinse solution for each test trough + 10 mL in excess.
2. Prepare Conjugate solution by diluting concentrated streptavidin-alkaline phosphatase conjugate (C) 1:100 in Conjugate Diluent (CD). Prepare 2 mL Conjugate solution for each test trough + 2 mL in excess.
3. Wash each strip twice for 1 min using 2 mL of the diluted Rinse solution.
4. Add 2 mL of the diluted Conjugate solution to each trough and incubate for 30 min while agitating the tray on the shaker.
5. Prepare Substrate solution by diluting the concentrated BCIP/NBT (bromochloroindolylphosphate/nitroblue tetrazolium) Substrate Solution (S) 1:100 in Substrate Buffer (SB). Prepare 2 mL substrate solution for each test trough + 2 mL in excess.
6. Wash each strip twice for 1 min using 2 mL of the diluted Rinse solution and wash once more using 2 mL Substrate Buffer (SB).
7. Add 2 mL of the diluted Substrate solution to each trough and incubate for 30 min while agitating the tray on the shaker.
8. Stop the color development by washing the strips twice in 2 mL distilled water while agitating the tray on the shaker for at least 3 min.

9. Using forceps, remove the strips from the troughs and place them on absorbent paper. Let the strips dry completely before reading the results. Store the developed and dried strips in the dark.

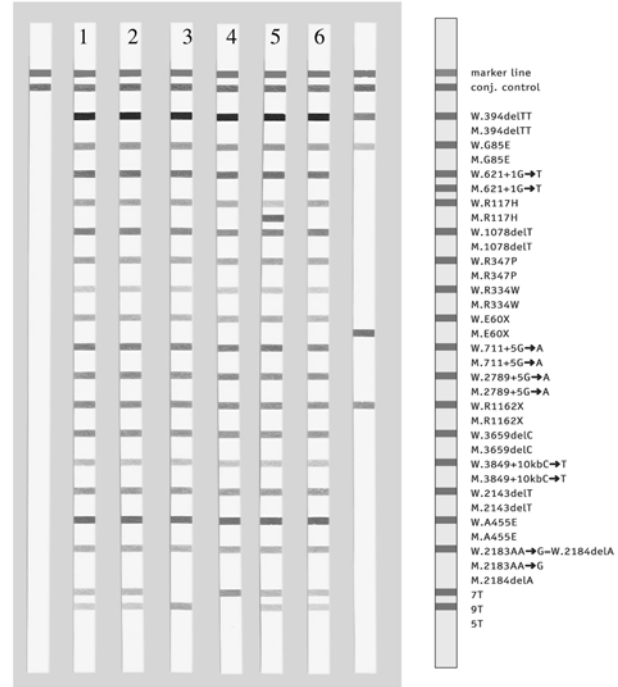
### 3.3.5. Evaluation

Examples of some readings are shown in **Fig. 6**. The plastic reading card should be used in order to find the position of the different allele-specific oligonucleotide probes on the strips. A line is considered positive when a clear purple/brown band appears at the end of the test procedure. The uppermost line on the strip is the marker line (black line). This line allows correct orientation of the strip. The next line controls the addition of reactive Conjugate solution and Substrate solution during the detection procedure. This line should be lined up with the Conjugate control line on the plastic reading chart. This line should always be positive, and should have approximately the same intensity on each strip in the same test run. The assay result of each negative control should give no apparent signal for any of the lines on the strip, except for the Conjugate control line. The assay result is valid when for each described mutant locus either the W-type probe, the M-type probe, or both probes are positive (the wild-type and mutant-type probes may differ in color intensities), except for successive mutation positions F508del/I507del and 2183AA→G/2184delA (**Note 4**). Individuals who are homozygous for a certain wild-type allele show a positive signal only for the corresponding wild-type probe, and not for the corresponding mutant-type probe. Individuals who are homozygous for a mutant allele show a positive signal only for the corresponding mutant-type probe, and not for the corresponding wild-type probe. Heterozygotes or compound heterozygotes show positive signals for both the corresponding wild-type and mutant-type probes. The oligonucleotide sequences of W.G551D, W.Q552X, and W.R553X overlap, which may result in aberrant reaction patterns from samples obtained from CF patients who are homozygous or compound heterozygous for these mutations—e.g., a weak signal for W.R553X for a patient who is compound heterozygous for G551D and R553X. Taking into account these patterns, correct interpretation is still possible. Whenever an abnormal hybridization pattern is observed, further investigation with alternative techniques such as sequencing should be considered. At least the mutations 621 + 2T > C, 621 + 3A > G, R170H, 711 + 1G > T, 711 + 3A > G, R347H, I506M, I506V, I507V, F508C, 1717-8G→A, 1717-2A→G, R560K, 2184insA, 2789 + 3delG, 3601-111G > C, R1162L, W1282R, and R1283M have been shown not to result in crossreactivity. For the Tn locus, if only one positive band (5T, 7T, or 9T) is seen, the patient will be homozygous for the respective allele. If two positive bands are seen, the patient will be compound heterozygote for these two respective alleles.

### INNO-LiPA CFTR12



### INNO-LiPA CFTR17+Tn



#### Results obtained with INNO-LiPA CFTR12 and INNO-LiPA CFTR17+Tn

Sample	CF-related mutations	Tn typing
CF00-1	DF508/-	7T/9T
CF00-2	G542X/-	7T/9T
CF00-3	DF508/G542X	9T/9T
CF00-4	G551D/R553X	7T/7T
CF00-5	DF508/R117H	7T/9T
CF00-6	N1303K/-	7T/9T

### 3.4. The OLA Cystic Fibrosis Assay

#### 3.4.1. DNA Preparation

1. Dilute each genomic DNA sample to a concentration of 500 ng/15  $\mu$ L in TE buffer (**Note 1**).
2. For each sample, plus a positive control and a negative control, add 60  $\mu$ L of buffer for purified DNA to a pre-labeled amplification tube.
3. Add 15  $\mu$ L of each DNA sample (15  $\mu$ L distilled water for the negative control) to the respective amplification tubes.
4. Mix thoroughly by gently vortexing.
5. Place the samples into the thermal cycler and heat for 40 min at 97°C and cool subsequently to 4°C. Upon completion, the samples may be stored at 2–8°C for 2 mo.

#### 3.4.2. PCR Amplification

1. For each sample, including the positive and negative control, pipet 5  $\mu$ L of CF PCR reagent into a pre-labeled amplification tube.
2. Remove a 5- $\mu$ L aliquot of each DNA preparation tube and add to the corresponding pre-labeled amplification tube.
3. Place the samples into the thermal cycler and perform a PCR using the following amplification profile: initial denaturation for 12 min at 94°C; denaturation for 15 s at 98°C, annealing for 30 s at 58°C, extension for 90 s at 72°C, for a total of five cycles; denaturation for 15 s at 94°C, annealing for 2 min at 58°C, for a total of 22 cycles; and finally, 5 min at 72°C, 30 min at 99.9°C and cooling to 4°C. On completion of the amplification program, the samples may be stored at 2–8°C for 1 mo.

#### 3.4.3. OLA Probing

1. Prepare an OLA mix by combining the entire contents of the CF OLA reagent tube with the entire contents of the rTth DNA ligase tube (for less than 24 assays, a smaller amount may be prepared). The OLA mix may be stored for 2 wk at 2–8°C, or for 3 mo at –15 to –25°C.
2. Pipet 10  $\mu$ L of OLA mix into each of the sample-containing amplification tubes. Mix by vortexing and quick-spin the samples.
3. Place the samples into the thermal cycler and perform the following cycling program: denaturation for 5 s at 90.5°C, annealing and ligation for 45 s at 46.5°C, for a total for 32 cycles, followed for 10 min at 99°C and subsequent cooling to 4°C. Upon completion, the samples may be stored at 2–8°C for 3 mo.

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Fig. 6. (*see opposite page*) Representative illustration of results obtained with the INNO-LiPA *CFTR* assays.

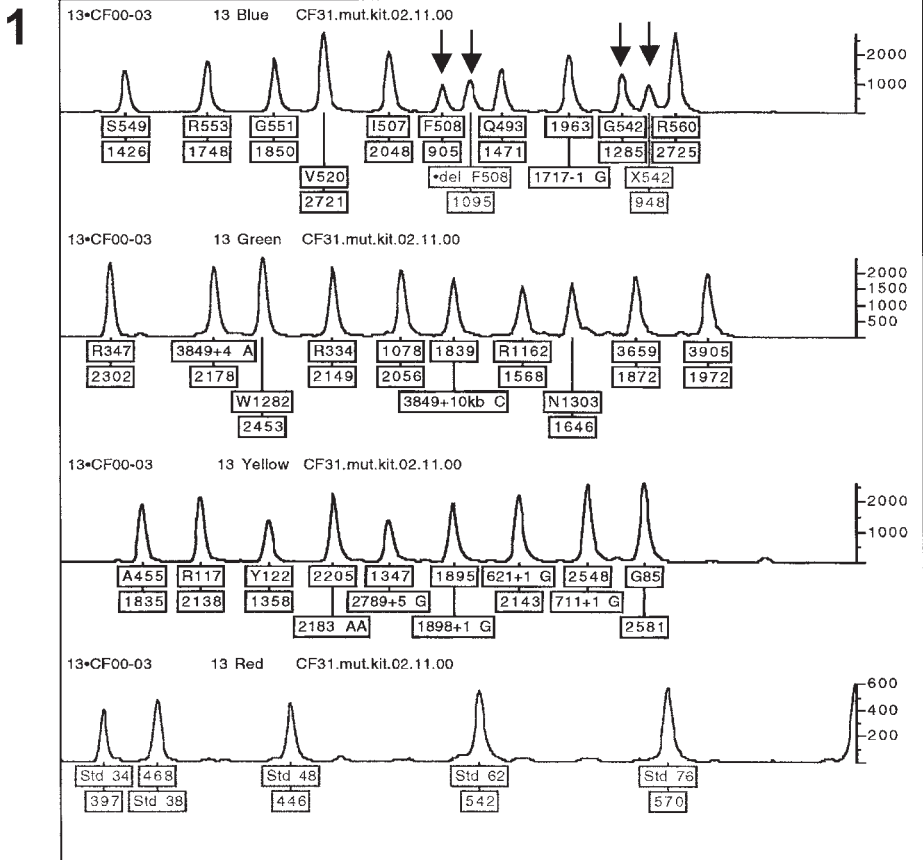


Fig. 7. Representative illustration of results obtained with OLA CF assay. Sample 1 is derived from an individual who is compound heterozygous for the F508del and G542X mutations.

#### 3.4.4. Electrophoresis

An aliquot of each sample is mixed with OLA-TAMRA size standard, formamide (loading buffer), denatured (2 min at 95°C and put on ice), and run on an ABI PRISM® 310 Genetic Analyzer, ABI PRISM™ 373 DNA Sequencer, or ABI PRISM® 377 DNA Sequencer, according to the recommendations of the manufacturer.

#### 3.4.5. Evaluation

The GeneScan® Analysis Software (according to the recommendations of the manufacturer) analyzes the data collected on the Genetic Analyzer (DNA



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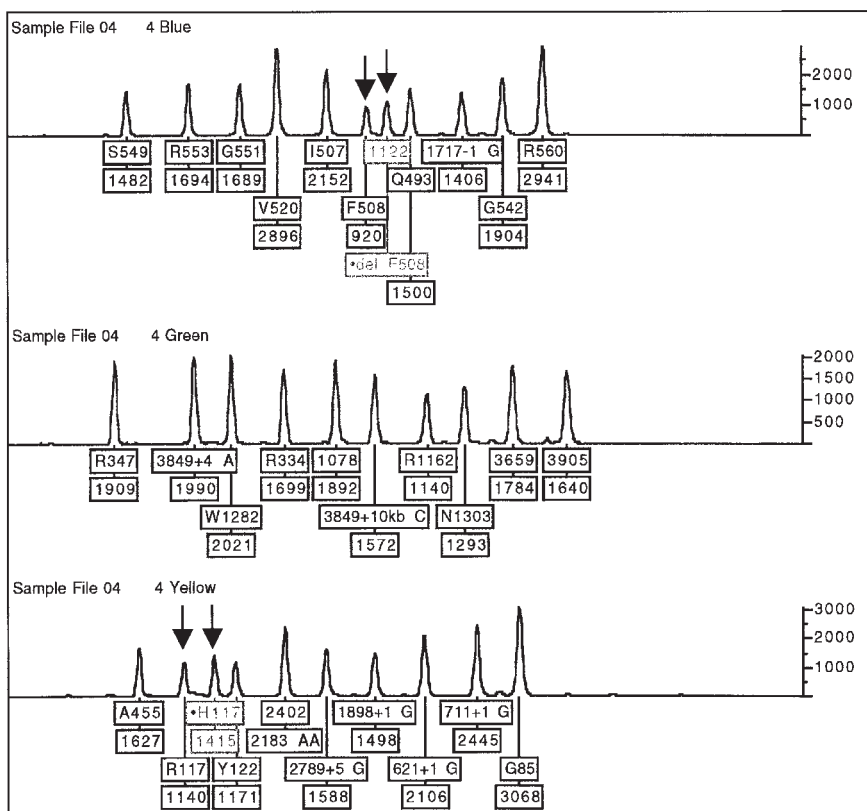


Fig. 7. (continued) Sample 2 is derived from an individual who is compound heterozygous for the F508del and R117H mutations.

Sequencer) to size and quantify the different fragments. A fluorescently labeled size standard is utilized by GeneScan<sup>®</sup> Analysis Software to size the OLA products. Identification of amplicons is accomplished by size separation of the amplicons in each of three colors. Genotyper<sup>®</sup> software (according to the recommendations of the manufacturer) interprets the data and generates the preliminary results for all loci tested—i.e., assignment of a *CFTR* allele to each peak and determination of the height of each peak. The height is important for determining the homozygous or heterozygous state of the corresponding allele. Representative examples are shown in Fig. 7. The results are then validated to confirm the *CFTR* genotype for each sample. All the size-standard peaks for each sample must be present. The negative control sample should have zero peaks; the positive control should have 29 normal peaks. For each sample, it is essential to check that there are no false peaks assigned because of raised base-

lines or dye-bleed-through (e.g., small peaks of a color that vertically align with large peaks of another color). Validated-heterozygous peak pairs must have peak heights that fall within a twofold variation of one another. A genotyped, heterozygous pair with greater than twofold variation in peak height must therefore be re-examined. The number of mutant peaks should be determined—count two mutations for each homozygous peak, and count one mutation for each heterozygous peak pair. There should be no more than two mutations per sample. Finally, the total peaks should be determined: 10–12 blue peaks, 10–12 green peaks, 9–11 yellow peaks (29–31 total peaks). If a different range of peaks is found, the sample should be re-examined. If the total number of peaks is 29, and the genotyped peaks are all normal, the sample is a wild-type homozygote for the tested mutations. If the total number of peaks is 29 and only one homozygous mutant *CFTR* allele occurs, and the other alleles are normal, the sample is a homozygous mutant for the respective mutation. If the total number of peaks is 30, and there is only one normal/mutant pair, and the other alleles are normal, the sample is heterozygous for the respective mutation. If the total number of peaks is 31, and there are two normal/mutant pairs, and the other alleles are normal, the sample is compound heterozygous for the respective mutations. Some mutations included in the assay can cause crossreactivity. In this regard, the mutations at S549, G551, and R553 are located within 14 basepairs of each other. Because of the number of mutations in this area, the probes are overlapping. In case of an abnormal outcome, such as diminishment in height of peak or absence of peak, the sample should be re-examined by another method. The F508C mutation has been shown to not crossreact in the assay.

#### 4. Notes

1. Rather than using highly purified DNA, DNA obtained from quick purification DNA protocols, of which only limited amounts of DNA are isolated, may be used. For the INNO-LiPA *CFTR* assays, a protocol for the isolation of DNA from buccal brushes is provided. For the Elucigene™ CF assays, a protocol for the isolation of DNA from mouthwash samples is provided. For the OLA CF assay, blood is used as such in a slightly modified DNA preparation protocol. For the DNA preparation, 15  $\mu$ L of blood (anticoagulated using EDTA) is added to 60  $\mu$ L of buffer for EDTA blood (instead of using the buffer for purified DNA). This buffer for EDTA blood is included in the kit. After heating at 97°C and cooling to 4°C, a centrifugation for 10 min at 14,000 rpm is performed, and the supernatant is used in subsequent steps.
2. The heteroduplex assay is a very robust assay. Most primer pairs that cover the deletion will allow the identification of the deletion by heteroduplex analysis.
3. The hybridization, stringent wash, and color development steps can be performed automatically with the *Auto*-LiPA apparatus. The *Auto*-LiPA is a walk-away

system with automated heating and cooling, and with automated aspiration and pipetting.

4. The wild-type sequence for I507del is identical to the wild-type sequence for F508del. A homozygous I507del/I507del or compound heterozygous F508del/I507del CF patient will therefore show no signal with the wild-type F508del probe. Similarly, the same wild-type sequence is used for the 2183AA→G, 2184delA mutations on the INNO-LiPA *CFTR*17 + Tn strip.

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## Familial Adenomatous Polyposis

Fiona Macdonald

### 1. Introduction

Familial adenomatous polyposis (FAP) is an autosomal, dominantly inherited disorder that predisposes to the development of hundreds to thousands of adenomatous polyps throughout the colon and rectum (MIM 175100). Left untreated, it is almost 100% certain that at least one of these polyps will become malignant, usually by the time the patient is 40 yr of age (**1**). Affected patients may also develop a range of extracolonic manifestations, including benign lesions such as congenital hypertrophy of the retinal epithelium (CHRPE), jaw cysts, and sebaceous cysts as well as desmoid tumors and other malignancies including periampullary tumors, thyroid cancers, adrenocortical tumors, brain tumors, and hepatoblastomas (**1**). The colorectal features, in association with extra colonic manifestations, are also known as Gardner's syndrome. A small proportion of patients have an attenuated form of the disease characterized by less than 100 polyps and a later age of onset, known as attenuated adenomatous polyposis coli (AAPC) (**2**). Hereditary desmoid disease is a relatively rare condition characterized by autosomal dominant inheritance of multiple desmoid tumors but with no colorectal polyposis (**3**). In general, the clinical features of the disorder allow a diagnosis of FAP to be made relatively easily. However, the attenuated form of the disease can show similarities to hereditary non-polyposis colon cancer (HNPCC), and mutation analysis of the causative genes may be required to aid in the differential diagnosis.

FAP is caused by constitutional mutations in the adenomatous polyposis coli (APC) gene. The gene comprises 8535 basepairs (bp) of coding sequence translated into a 2843 amino acid protein in its most common isoform (**4,5**). Mutation analysis is directed at 15 exons, although an additional six exons are found in

alternatively spliced forms of the transcript. Mutations occur throughout the gene, although the majority of mutations are localized in exon 15 (6–8). This exon comprises more than 75% of the coding sequence of APC. There are two relatively common mutations in exon 15, which together account for about 10% of cases worldwide. These are both 5-bp deletions that occur at codons 1061 and 1309. The majority of mutations are non-sense or frameshift mutations leading to a truncated protein product. The role of missense changes remains unclear, and although some of them are considered as potentially low penetrance mutations, their interpretation in the diagnostic setting is difficult (6,9).

Some genotype-phenotype correlations exist that can be helpful in indicating the region of the gene in which mutations are located. CHRPEs are only present in patients with mutations between codons 457 and 1444 (10–12). Desmoid tumors usually occur in patients with 3' mutations, particularly between codons 1403 and 1558 (3,6,12). In addition, extracolonic manifestations such as epidermoid cysts and osteomas also occur primarily in patients with mutations in this region (6). AAPC is associated with mutations at the 5' or 3' ends of the gene or with mutations in the alternatively spliced region of exon 9 (2,13). A severe phenotype is particularly associated with mutations between codons 1250 and 1464, and especially with the 5-bp deletion mutation at codon 1309 (14). Care should be taken in using these correlations for any purpose other than indicating where to start mutation analysis as they are not absolute and should not be used when reporting to indicate the probable phenotype of the patient.

### 1.1. Strategy

Samples are referred to diagnostic laboratories for three purposes: for mutation analysis of affected individuals, for presymptomatic testing in at-risk family members once a mutation has been identified in the family, and for linkage analysis if a mutation has not been identified.

#### 1.1.1. Mutation Analysis in Affected Individuals

Mutations have been detected in approx 80% of individuals with clinically diagnosed FAP. In general, exon 15 is analyzed by the protein truncation test (PTT) in four overlapping reactions (segments 2–5; 15). Exons 1–14 can also be analyzed by PTT (segment 1), but given the need for RNA as the starting material and the presence of alternate splicing, it is not a commonly used procedure. Exons 3–14 can be analyzed by single-stranded conformational polymorphism analysis (16: SSCP), heteroduplex analysis (17: HA), and denaturing gradient gel electrophoresis (DGGE) (6,7,10). Both RNase protection and chemical cleavage of mismatches have also been used to investigate APC mutations, although they are technically more complex and are often used in labo-

ratories with experience in these methods. Most diagnostic laboratories use either SSCP or DGGE; thus, only these two techniques will be described for analysis of the first 14 exons. Mutations have not been detected in exons 1 or 2, and these exons are rarely investigated. Exon 15 can also be analyzed by the same methods in approx 23 overlapping segments. This is obviously a more time-consuming approach than PTT, but it may be useful in laboratories not set up to carry out PTT. It is also possible to look directly for the two common 5-bp deletion mutations at codons 1061 and 1309, by polymerase chain reaction (PCR) using primers flanking the deletion, particularly if laboratories only intend to carry out a preliminary screen. However, both these mutations can be detected by PTT/SSCP/DGGE.

In the absence of colonic and extracolonic clinical details, lymphocyte DNA samples from FAP-affected individuals are first tested for the presence of chain-terminating mutations in segments 2, 3, and 4 of exon 15 by the PTT. For samples with no detectable mutation, exons 3–14 are then systematically tested by SSCP/DGGE. The distribution of mutations in these exons is shown in **Table 1**. Data is shown from the West Midlands Regional Genetics Service, and includes results primarily from UK families and, for comparison, from a worldwide database. In the first instance, it is possible to target analysis to those exons most often found to contain mutations. If no mutations are found in any of these exons, analysis of segment 5 of exon 15 by PTT should be carried out. As mutations are rarely found in this region (**Table 1**), PTT mutations in other regions can be excluded after SSCP analysis of the rest of the gene has been carried out.

If the phenotype of patients is known, a more targeted screening strategy can be adopted. Samples from patients known to be CHRPE-negative should initially be screened for mutations in APC exons 3–8 by SSCP/DGGE, followed by screening of segment 4 of exon 15 by PTT. If samples analyzed in this initial screen have no identifiable mutations, it is still important to screen the remainder of the gene, as detection of CHRPE is not always 100% accurate. Exons 9–14 should then be screened by SSCP/DGGE and segments 2, 3, and 5 should be analyzed by PTT. If a patient has multiple extra colonic features, segments 2, 3, and 4 of exon 15 should be analyzed initially by PTT and if no mutations are found the remainder of the gene should then be studied. Patients with attenuated APC should be screened for mutations in exons 3, 4, or 9 by SSCP/DGGE and by PTT analysis of segment 4. Other regions of the gene should then be investigated if no mutations are detected. In all situations, any variants identified either by SSCP/DGGE or by PTT should always be characterized by sequencing analysis to confirm the nature of the mutation.

If a mutation has not been detected by any of these methods, it is worth considering the possibility of a large deletion of all or part of the APC gene. Evidence increasingly suggests that deletions may account for up to 5% of cases

**Table 1**  
**Distribution of APC Mutations in a UK Laboratory Population**  
**and on an International Database**

Exon or segment (of exon 15)	Distribution of mutations in 201 families from a UK laboratory (percentage of total)	Distribution of mutations on a worldwide database* (percentage of total)
Exon 3	2 (0.9)	8 (0.5)
Exon 4	6 (2.9)	20 (1.2)
Exon 5	4 (1.9)	19 (1.1)
Exon 6	7 (3.4)	27 (1.7)
Exon 7	5 (2.4)	9 (0.6)
Exon 8	14 (6.9)	34 (2.1)
Exon 9	12 (5.9)	19 (1.1)
Exon 10	4 (1.9)	7 (0.4)
Exon 11	9 (4.4)	19 (1.1)
Exon 12	5 (2.4)	10 (0.6)
Exon 13	4 (1.9)	29 (1.8)
Exon 14	13 (6.4)	40 (2.5)
Segment 2 <sup>#</sup>	68 (33.8)	399 (25)
Segment 3 <sup>#</sup>	48 (23.8)	910 (57)
Segment 4 <sup>#</sup>	3 (1.4)	28 (1.7)
Segment 5 <sup>#</sup>	0 (0)	9 (0.6)

\*Data taken from APC database at <http://perso.curie.fr/Thierry.Soussi/APC.html>

<sup>#</sup>If mutations were detectable in overlapping regions of two segments, they were counted in the first segment in which they could be seen. They were not counted in the second segment.

of UK FAP families (*18*). These deletions can be detected by quantitative fluorescence PCR (QF-PCR). Finally, a few patients may have a deletion large enough to be detected by cytogenetics. These patients may exhibit other symptoms, such as mental retardation caused by deletions of genes in addition to APC.

A list of published APC mutations and polymorphisms can be found at <http://perso.curie.fr/Thierry.Soussi/APC.html>.

### 1.1.2. Presymptomatic Analysis

Because FAP is an autosomal dominant condition, all offspring of an affected parent are at 50% prior risk of developing the condition. Before the introduction of molecular testing, all individuals had to be monitored annually by sigmoidoscopy from their early teens until the fifth decade. However, now that



mutation analysis or linkage can identify gene carriers, only those individuals identified to be at high risk need to be monitored closely and offered surgical intervention before the development of a cancer.

Presymptomatic analysis should only be offered once a mutation has been identified in a family or once linked markers have been shown to be informative. Ideally, testing should be carried out when possible, using two different methods to look for the mutation. Some mutations may introduce or remove a restriction site so that a direct test can be used, as well as sequencing of the region that contains the mutation. Alternatively, SSCP/DGGE/PTT can be used followed by sequencing. If this is not possible, testing should at least be done by two separate sequencing reactions or two analyses of the linked marker. Individuals who do not carry the familial mutation are then at population risk of developing colorectal cancer, and do not need to undergo regular invasive screening.

### **1.2. Linkage Analysis**

If mutation analysis has not detected a mutation, or if a rapid result is required while mutation analysis is ongoing, it is possible to carry out linkage analysis in suitable families. The definite diagnosis of FAP is needed before this approach is taken, and DNA must be available from affected individuals. If there are no living affected individuals, it is possible to obtain DNA from fixed tissue in the form of paraffin blocks from deceased individuals as an alternative (19). Approximately 20% of FAP cases are the result of new mutations, so linkage analysis is not possible if there is only an isolated case in the family. Although the majority of cases of FAP have been shown to be caused by the APC gene at 5q21, there have been rare reports of cases of families who do not show linkage to this locus, and this must be taken into account when issuing reports (20).

There are a number of highly informative polymorphic microsatellite markers linked to the APC gene, which can be used to generate a high-risk haplotype (*see Table 1*). There are also several intragenic markers that can be used, although these are all di-allelic and are not always informative. An additional problem is that these markers are in disequilibrium, so if one is uninformative, the others may be similar. To reduce errors from recombination, families should be made informative with flanking markers as well as an intragenic marker wherever possible.

## **2. Materials**

Analytical-grade reagents should be used at all stages unless otherwise specified. The majority of materials used are described in the chapters detailing the methodology. Only relevant details that are specific to FAP are provided here.

### 2.1. PTT Analysis

1. Deoxynucleotide triphosphates (dNTPs). Dilute individual dNTPs (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) in distilled water to give a stock mixture of 2 mM.
2. Taq polymerase and PCR buffer: The author uses the Invitrogen (Paisley, UK) enzyme, 10X buffer, and 50 mM magnesium chloride supplied with it.
3. Oligonucleotide primers: These can be obtained from a number of commercial companies such as Invitrogen or Sigma (Poole, Dorset, UK). They should be diluted to yield a concentration of 25  $\mu$ M for use. Details of the primers used are given in **Table 2**.
4. TNT T7 quick transcription/translation kit (Promega, Southampton, Hampshire).
5.  $^{35}$ S Methionine (1000Ci/mmol; Amersham Pharmacia Biotech).
6. Acrylamide: 40% ready-mixed solution at a 29:1 acrylamide/*bis* ratio (Sigma).
7. Electrophoresis system: The mini Protean II system (Biorad, Hemel Hempstead, Hertfordshire, UK) works well for PTT analysis of APC.

### 2.2. DGGE Analysis of Exons 3–14

1. PCR reagents used are as described for PTT.
2. Oligonucleotide primers: Sources and concentrations as for PTT. Details of primers used are given in **Table 3**.
3. Acrylamide: Prepare stock denaturant solutions containing 80% and 0% denaturant as shown in **Table 4** and store at 4°C.
4. DGGE electrophoresis system: e.g., Ingeny system (Genetic Research Instrumentation, Braintree, Essex, UK).

### 2.3. SSCP Analysis

1. PCR reagents used are as described for PTT.
2. Oligonucleotide primers: Sources and concentrations as for PTT. Details of primers used are given in **Table 5**.
3. Acrylamide: 40% ready-mixed solution at a 49:1 acrylamide/*bis* ratio (Sigma).
4. Electrophoresis system: Model SA32 system 32  $\times$  20 cm (Invitrogen).
5. Silver staining solutions: i) 10% ethanol; ii) 1% nitric acid; iii) 0.1% silver nitrate (0.5 g silver nitrate in 500 mL distilled water. This solution should be stored at room temperature, either in a brown bottle or bottle covered with silver foil, as solution is light-sensitive); iv) 0.28 M sodium carbonate, 0.019% formalin; v) 10% acetic acid.
6. Cellophane sheets: Promega (Southampton, Hampshire, UK).
7. Drying frame: Promega (Southampton).

### 2.4. Analysis of 5-bp Deletion Mutations in Exon 15 Codons 1061 and 1309

1. PCR reagents used are as described for PTT.
2. Oligonucleotide primers: Sources and concentrations as for PTT. Details of primers used are given in **Table 6**.

**Table 2**  
**Sequence of Primers for PTT Analysis of APC Exon 15 Segments 2–5**

Segment	Codons of APC	Sequence of forward primer (from 5' to 3')	Sequence of forward primer (from 5' to 3')	Annealing temperature (°C)
2	654 to 1263	TAATACGACTCACTATAGGAACAGACCA CCATGCAAATCCTAAGAGAGAACAACCTGTC	CACAATAAGTCTGTATTGTTTCTTGG	58
3	990 to 1700	TAATACGACTCACTATAGGAACAGACCA CCATGGATGAAAGTAAGTTTTGCAGTTATGG	GAGCCTCATCTGTACTTCTGC	58
4	1547 to 2339	TAATACGACTCACTATAGGAACAGACCA CCATGGAAAACCAAGAGAAAAGAGGCAG	GAGGACTTATTCCATTTCTACC	55
5	2131 to 2844	TAATACGACTCACTATAGGAACAGACCA CCATGTGGAAAGCTATTCAGGAAGGTG	CCAGAACAAAAACCCCTCTAACAAG	55

Primers from (15) and from author's laboratory.

**Table 3**  
**Sequence of Primers for DGGE Analysis of APC Exons 3–14**

Exon	Sequence of forward primer (from 5'–3') (1–5 indicates the GC clamp to be added)	Sequence of reverse primer (from 5'–3') (1–5 indicates the GC clamp to be added)	Annealing Temperature (°C)	Gradient of denaturant for DGGE analysis
3p	3- CAATTGTTGTATAAAAACTTGTT	CCATAAGAACGGAGGGACA	55	20–70
3m	3- GTAAAAC TGCGGTCAAAA	GAACCCATAGGAACAGGACT	55	20–70
3d	ATCTGTATCAAGCCGTITCTG	2- AATGTTTACTATATGAAGAAAAG	55	20–70
4	3- ATAGGTCATTGCTTCTTGC	TGAATTTTAATGGATTACCTAGGT	55	20–70
5	TTCTTTATTGGTTCTTATATGC	1-TGTAATTCATTTTATTCCTAATAGCTC	55	20–50
6	1- GGTTTCTTGTTTTATTITTAGCG	CTACCTATTTTATAACCAC	58	20–70
7	1- AAGAAAGCCTACACCATTTTGC	GATCATTCTTAGAACCATCTTGC	55	30–60
8	CATGATGTTATCTGTATTTACC	1- CTTAGCAAAGTAGTCATGGC	58	30–60
9p	AGTCGTAATTTGTTTCTAAACTC	4- AGCCTCTTACTGCCCCG	55	20–70
9d	5- CCTCTCCTCATCCAGCTTTTAC	GCTTTGAAACATGCACTACGATG	55	40–80
10	1- CATCATTGCTCTTCAAATAAC	CACCAGTAATTGTCTATGTCA	58	30–60
11	TAGATGATTGTCTTTTTCTCTTG	1- TCATACCTGAGCTATCTTAAG	55	20–70
12	3- AAGCTTGGCTTCAAGTTGTC	CAGAGTGAGACCCTGCCTC	58	20–70
13	GCAACTAGTATGATTTTATGTATAAA	1- ACATGAAATTCATATTATAGTACT	55	20–50
14	CAACTCTAATTAGATGACCCA	2- CTTTAAAAGTAATATAAAACTC	55	20–70

GC clamp sequence

- 1- 5'CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCCGCCCC3'
- 2- 5'CCCCACGCCACCCGACGCCCCAGCCCGACCCCCCGCGCCCGGCGCCCC3'
- 3- 5'CCCCGCCC GCGCCCGCCCCGCCCCCGCCCCCTCCCGGCCGCCCCCTGGCGCCCCGC3'
- 4- 5'CCCCCCCCGCCCCGCGGTCCCCCTGCCCGGCCCTCCCCCGCCCCCCCCCGCCCC3'
- 5- 5'CGCCCCGCCCCGCCCCCGCCCCCGCCCCCTCCCGGCCGCCCCCTGGCGCCCCGCCCCCCCC3'

Primers from (6,7,10)

**Table 4**  
**Stock Solutions for DGGE Analysis**

	0% denaturant	80% denaturant*
40% acrylamide/ <i>bis</i> -acrylamide (19:1)	100 mL	100 mL
20X TAE	25 mL	25 mL
Deionized formamide <sup>#</sup>	None	160 mL
Urea	None	170 g
Distilled water	Up to 500 mL	Up to 500 mL

\*32% formamide/5.6 M urea

<sup>#</sup>To deionize formamide:

1. Add 3 g mixed bed resin (Biorad) per 100 mL formamide.
  2. Mix for at least 30 min at room temperature in a fume hood.
  3. Filter the mixture through Whatman paper.
  4. Store deionized formamide at 4°C in a light-resistant bottle.
3. Acrylamide: 40% ready mixed solution at a 19:1 acrylamide/*bis* ratio (Accugel, National Diagnostics, Hull, Humberside, UK).
  4. Electrophoresis system: Mini Protean II (Biorad).

### 2.5. Sequencing Analysis

1. Shrimp alkaline phosphatase/exonuclease 1 (Amersham Pharmacia Biotech).
2. Sequencing is carried out in the majority of laboratories using fluorescent-based cycle sequencing—e.g., with D-rhodamine or big dye kits from Applied Biosystems (ABI) (Warrington, Cheshire, UK). However, the specific method used is dependent on the sequencer available in the user’s laboratory (*see Note 1*).
3. Oligonucleotide primers: The primers used for sequencing as listed in **Table 3** for DGGE, but minus the GC clamp or as for SSCP analysis in **Table 5**.
4. Electrophoresis system: automated sequencer.

### 2.6. Linked and Intragenic Marker Analysis

1. PCR reagents used are as described for PTT.
2. Oligonucleotide primers: Sources and concentrations as for PTT. Details of primers used are shown in **Table 7**. Additional details of the markers are given in **Table 8**. Primers for linked markers should be labeled with the fluorochrome appropriate to the sequencer used (*see Note 2*).
3. Electrophoresis system: automated sequencer for fluorescently labeled markers, agarose gel electrophoresis for intragenic markers.

**Table 5**  
**Sequence of Primers for SSCP Analysis of APC**

Exon	Sequence of forward primer (from 5' to 3')	Sequence of reverse primer (from 5' to 3')
3	TAACCTAGATAGCAGTAATTTCCC	ACAATAAACTGGAGTACACAAGG
4	ATAGGTCATTGCTTCTTGCTGAT	TGAATTTTAATGGATTACCTAGGT
5	CTTTTTTTGCTTTTACTGATTAACG	TGTAATTCATTTTATTCCTAATAGCTC
6	GGTAGCCATAGTATGATTATTTCT	CTACCTATTTTTTATACCCACAAAC
7	AAGAAAGCCTACACCATTTTTGC	GATCATTCTTAGAACCATCTTTCG
8	ACCTATAGTAATTATACCATC	GTCATGGCATTAGTGACCAG
9	AGTCGTAATTTGTTTCTAAACTC	TGAAGGACTCGGATTTACCGC
9A	TCATTCACTCACAGCCTGATGAC	GCTTTGAAACATGCACTACGAT
10	AAACATCATTGCTCTTCAAATAAC	TACCATGATTTAAAAATCCACCAG
11	GATGATTGTCTTTTTCTCTTGC	CTGAGCTATCTTAAGAAATACATG
12	TTTTAAATGATCCTCTATTCTGTAT	ACAGAGTCAGACCCTGCCTCAAAG
13	TTTCTATTCTACTGCTAGCATT	ATACACAGGTAAGAAATTAGGA
14	TAGATGACCATATTCTGTTTC	CAATTAGGTCTTTTTGAGAGTA
15A	GTTACGCATACACATTGTGAC	GCTTTTTGTTTCCTAACATGAAG
15B	AGTACAAGGATGCCAATATTATG	ACTTCTATCTTTTTCAGAACGAG
15C	ATTTGAATACTACAGTGTTACCC	CTTGATTTCTAATTTGGCATAAGG
15D	CTGCCCATACACATTCAAACAC	TGTTTGGGTCTTGCCCATCTT
15E	AGTCTTAAATTCAGATGAGCAG	GTTTCTTCATTATATTTTATGCTA
15F	AAGCCTACCAATTATAGTGAACG	AGCTGACAAAGATGATAATG
15G	AAGAAACAATACAGACTTATTGTG	ATGAGTGGGGTCTCCTGAAC
15H	ATCTCCCTCCAAAAGTGGTGC	TCCATCTGGAGTACTTTCTGTG
15I	AGTAAATGCTGCAGTTCAGAGG	CCGTGGCATATCATCCCC
15J	CCCAGACTGCTTCAAATAATACC	GAGCCTCATCTGTACTTCTGC
15K	CCCTCCAAATGAGTTAGCTGC	TTGTGGTATAGTTTTACTGGTG
15L	ACCCAACAAAAATCAGTTAGATG	GTGGCTGGTAACTTTAGCCTC
15M	ATGATGTTGACCTTTCCAGGG	ATTGTGTAACTTTTCATCAGTTGA
15N	AAAGACATACCAGACAGAGGG	CTTTTTTGGCATTGCGGAGCT
15O	AAGATGACCTGTTGCAGGAATG	GAATCAGACGAAGCTTGTCTAGAT
15P	CCATAGTAAGTAGTTACATCAAG	AAACAGGACTTGTACTGTAGGA
15Q	CAGCCCCTTCAAGCAAACATG	GAGGACTTATCCATTCTACC
15R	CAGTCTCCTGGCCGAAACTC	GTTGACTGGCGTACTAATACAG
15S	TGGTAATGGAGCCAATAAAAAGG	TGGGAGTTTTCGCCATCCAC
15T	TGTCTCTATCCACACATTTCGTC	ATGTTTTTCATCCTCACTTTTTGC
15U	GGAGAAGAAGTGAAGTTCATC	TTGAATCTTAAATGTTTGGATTTCG
15V	TCTCCACAGGTAATACTCCC	GCTAGAAGTGAATGGGGTACG
15W	CAGGACAAAATAATCCTGTCCC	ATTTTCTTAGTTTCATTCTCCTC

Primers from (16)

**Table 6**  
**Sequence of Primers for Direct Detection of 5-bp Deletions**  
**at Codons 1061 and 1309**

Codon	Sequence of forward primer (from 5' to 3')	Sequence of reverse primer	Annealing temperature (°C)
1061	GAATGAAAGATGGGCAAGACC	GATTCCTTGATTGTCTTGC	55
1309	GACACAGGAAGCAGATTCTG	TTCGCTCACAGGATCTTCAG	58

Primers from (6).

### 3. Methods

#### 3.1. PTT Analysis

##### 3.1.1. PCR Conditions

The final concentration of components are: 1X Taq buffer, 2 mM magnesium chloride, 200  $\mu$ M dNTP mix, 1  $\mu$ M of each primer, 2 U of Taq polymerase and 500  $\mu$ g of DNA in a 25- $\mu$ L reaction (*see Note 3*).

1. Prepare a master mix with all components except DNA. Aliquot 24  $\mu$ L into each PCR tube.
2. Add 1  $\mu$ L of DNA or 1  $\mu$ L distilled water for blank control into each tube.
3. Overlay with mineral oil (if necessary).
4. PCR conditions are 95°C for 4 min initial denaturation followed by 35 cycles of 95°C for 30 s, X°C for 30 s (specific annealing temperatures are provided in **Table 2**), 72°C for 2 min. This is followed by a single synthesis step of 72°C for 10 min.
5. Check the efficiency of the PCR by electrophoresis of 5  $\mu$ L of PCR product on a 1% agarose gel.
6. Samples can be stored at 4°C at this stage until required for PTT analysis.

##### 3.1.2. PTT Analysis

1. Components of the reaction are 1.5  $\mu$ L of PCR product, 0.3  $\mu$ L of 50 mM magnesium chloride, 0.7  $\mu$ L of nuclease-free water, 0.5  $\mu$ L of <sup>35</sup>S methionine, 9.5  $\mu$ L transcription/translation mix.
2. Incubate at 30°C for 90 min.
3. Add 50  $\mu$ L sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.
4. Store at -20°C until ready for electrophoresis.

**Table 7**  
**Sequence of Primers for Microsatellites and Intragenic Markers**

Marker	Sequence of forward primer	Sequence of reverse primer	Annealing temperature (°C)
CB26	*GTAAGCAGGACAAGATGACAG	GCTATTCTCTCAGGATCTTG	57
YN5.64	*CCCAATTGTATAGATTTAGAAGTC	TGAGGGCCAACGGATTATGA	58
CB83	*GTAGGGGTAAATTAGCCTCTC	GATAATGGAGGAGGCTATGC	57
LNS	*ACTCACTCTAGTGATAAATCGGG	AGCAGATAAGACAAGTATTACTAGTT	58
MBC	*GTGTTGGCAATGCAGTAGTG	ACCACTGGACTCCAGCCTGG	62
CA25	*TCTAGAGGATCTTCCCTCTT	CCATCGGTAGAACTAATTTT	52
5'UTR/DdeI	GAGCTGCGGACCGAGG	CCCAGGGAGGCGTACATA	58
Exon 11/RsaI	GATGATTGTCTTTTTCTCTTGC	CTGAGCTATCTTAAGAAATACATG	55
Exon 15/MspI	ATGATGTTGACCTTTCCAGGG	CTTTTTTGGCATTGCGGAGCT	60
3'UTR/SspI	GCATTAAGAGTAAAATTCCTCTTAC	ATGACCACCAGGTAGGTGTATT	55

\*fluorescent label if analysis is to be carried out on an automated sequencer.



**Table 8**  
**Additional Information on Microsatellite Markers**

Marker	Locus	Location relative to APC	Recombination with APC	PIC	Allele Size (bp)	GDB Id*
CB26	D5S299	Proximal	8%	0.66	156–182	185754
YN5.64	D5S82	Proximal	4%	0.70	169–179	180445
CB83	D5S122	Proximal	2%	0.19	211,213	180444
LNS	D5S346	Distal	<1%	0.83	96–106	181171
MBC	MCC gene	Distal	<1%	0.49	168–176	181466
CA25	D5S318	Distal	5%	0.78	116–128	186851

\*Accession reference in the Genome database ([www.gdb.org](http://www.gdb.org)).

### 3.1.3. SDS-PAGE

1. Prepare a 15% separating gel with a 5% stacking gel.
2. Denature the samples at 70°C for 10 min, then place on ice.
3. Load 15 µL of each sample onto the gel.
4. Electrophorese at 10–15 V/cm for approx 90 min or until the dye front has just left the bottom of the gel.
5. Fix the gel, dry, and expose to X-ray film.
6. Develop the autoradiograph after overnight exposure (**Fig. 1**). Any truncations identified should then be sequenced to confirm the nature of the truncating mutation (*see* **Notes 4** and **5**).

## 3.2. DGGE Analysis

### 3.2.1. PCR Conditions

The final concentration of components is: 1X Taq buffer, 2 mM magnesium chloride, 200 µM dNTP mix, 1 µM of each primer, 2 U of Taq polymerase, and 500 µg of DNA in a 25-µL reaction (*see* **Note 6**).

1. Prepare a master mix with all components except DNA. Aliquot 24 µL into each PCR tube.
2. Add 1 µL of DNA or 1 µL distilled water for blank control into each tube.
3. Overlay with mineral oil (if necessary).
4. PCR conditions are 95°C for 4 min, initial denaturation followed by 35 cycles of 95°C for 60 s, X°C for 60 s (specific annealing temperatures are provided in **Table 3**), 72°C for 60 s. This is followed by a single synthesis step of 72°C for 5 min.
5. Check the efficiency of PCR by electrophoresis of 5 µL of PCR product on a 1% agarose gel.

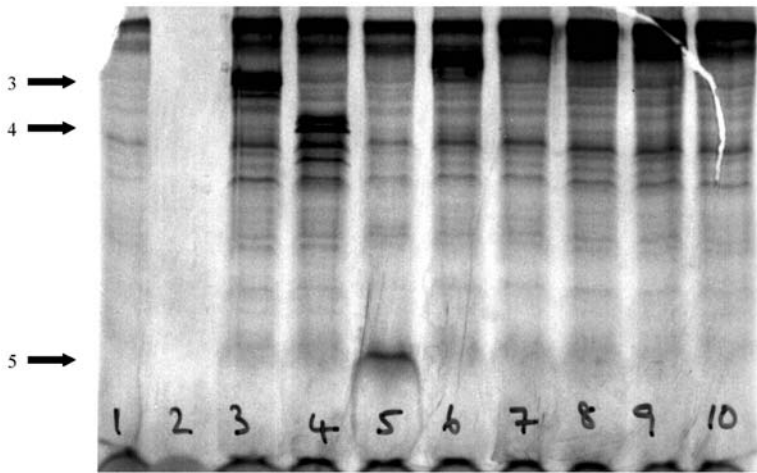


Fig. 1. PTT analysis of segment 4 of APC. Truncated products can be seen in lanes 3, 4, 5, and 6. A normal control sample is included in lane 1.

### 3.2.2. DGGE Analysis

1. Prepare a gel with the gradient appropriate to the exon under investigation. **Table 9** shows the volumes of 80% and 0% stock denaturant solutions needed to make the gradients specific for each exon as listed in **Table 3**. Add 140  $\mu\text{L}$  of 10% ammonium persulfate (freshly made) and 14  $\mu\text{L}$  of *N,N,N,N'* tetramethylethylenediamine (TEMED) to each denaturant solution prior to adding to gradient maker. Once poured, allow the gel to polymerize for at least 90 min.
2. While the gel is polymerizing, warm the TAE buffer in the tank to 60°C.
3. Pre run the gel at 100 V for 15 min prior to adding the samples.
4. Mix 7  $\mu\text{L}$  of each sample with 3  $\mu\text{L}$  of non-denaturing loading buffer and load the entire 10  $\mu\text{L}$  into the wells.
5. Electrophorese the gel at 5 V/cm for 16 h.
6. After the electrophoresis is finished, stain the gel with ethidium bromide to visualize the DNA (**Fig. 2**).
7. Any variants should be sequenced to confirm whether they are polymorphisms or mutations (*see* **Notes 6** and **7**).

### 3.3. SSCP Analysis

#### 3.3.1. PCR Conditions

The final concentration of components are: 1X Taq buffer, 1.5 mM magnesium chloride, 200  $\mu\text{M}$  dNTP mix, 1  $\mu\text{M}$  of each primer, 2 U of Taq polymerase, and 500  $\mu\text{g}$  of DNA in a 25- $\mu\text{L}$  reaction (*see* **Note 6**).

**Table 9**  
**Volumes of 0% and 80% Denaturant Solutions to Make Denaturing Gradient Gels Using INGENY PhorU System**

Final denaturant solution (%)	Volume 0% denaturant (mL)	Volume 80% denaturant (mL)*
20	20.5	7
30	17.1	10.4
40	13.8	13.8
50	10.4	17.1
60	6.8	20.7
70	3.4	24.1
80	0	27.5

\* *see Table 4.*

1. Prepare a master mix with all components except DNA. Aliquot 24  $\mu\text{L}$  into each PCR tube.
2. Add 1  $\mu\text{L}$  of DNA or 1  $\mu\text{L}$  distilled water for blank control into each tube.
3. Overlay with mineral oil (if necessary).
4. PCR conditions are 94°C for 3 min initial denaturation followed by 30 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. This is followed by a single synthesis step of 72°C for 5 min.
5. Check the efficiency of PCR by electrophoresis of 5  $\mu\text{L}$  of PCR product on a 1% agarose gel.

### 3.3.2. SSCP Analysis (see **Note 8**)

1. Pour an 8% polyacrylamide gel.
2. Place gel at 4°C for at least 1 h before running (see **Note 9**).
3. Mix 3  $\mu\text{L}$  of PCR product with 3  $\mu\text{L}$  of formamide-loading buffer. Denature the samples by heating at 95°C for 5 min, then snap-cool on ice.
4. Load the samples on to the gel and run at 10 V/cm overnight (approx 16 h) until the xylene cyanol dye is approx 5 cm from the foot of the gel (see **Note 10**).
5. After electrophoresis is complete, visualize the DNA by silver staining.

### 3.3.3. Silver Staining

1. Incubate the gel for at least 5 min in approx 300 mL of solution A with gentle shaking to fix the DNA (see **Note 11**).
2. Incubate the gel in 300-mL solution B for 10 min with gentle shaking to oxidize the DNA.
3. Rinse the gel in distilled water for 2 min.
4. Incubate the gel in 250 mL of solution C with gentle shaking for 15 min.

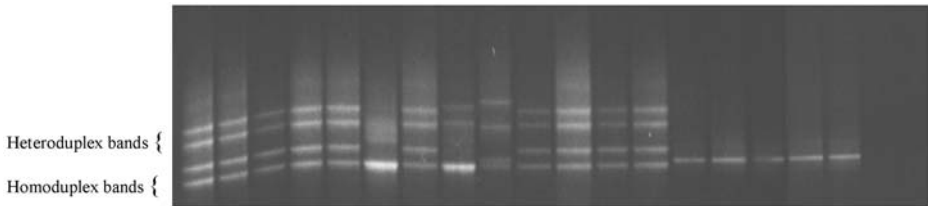


Fig. 2. DGGE analysis of exon 11 of APC, which contains a polymorphism. Lanes 1–5, 7, and 10–13 are heterozygous for the polymorphism, but do not show a variant. Lane 6 is homozygous for allele 2 of the polymorphism. Lane 9 shows allele 2 of the polymorphism and also shows a variant pattern. A variant in lane 8 can be seen only by the different patterns of the heteroduplexes.

5. Rinse again in distilled water for 2 min.
6. Incubate the gel in approx 300 mL of solution D (change the solution 2–3 times as soon as it turns gray). The DNA should be visible after the first solution change.
7. When the DNA is sufficiently dark and before the gel turns yellow, stop the reaction with 300 mL of solution E. This should be done in a fume hood. Shake gently for 5 min.
8. Rinse the gel in distilled water.
9. The gel may be dried on a gel drier or between two sheets of cellophane film in a drying frame at room temperature overnight (usually approx 16 h) (**Fig. 3**).
10. Any samples that show a band shift should be sequenced.

### **3.4. Analysis of 5-bp Deletion Mutations in Exon 15 Codons 1061 and 1309**

#### **3.4.1. PCR Conditions**

The final concentration of components is: 1X Taq buffer, 1.5 mM magnesium chloride, 200  $\mu$ M dNTP mix, 1  $\mu$ M of each primer, 2 U of Taq polymerase, and 500  $\mu$ g of DNA in a 25- $\mu$ L reaction (*see Note 6*).

1. Prepare a master mix with all components except DNA. Aliquot 24  $\mu$ L into each PCR tube.
2. Add 1  $\mu$ L of DNA or 1  $\mu$ L distilled water for blank control into each tube. Include a known positive control from a sample that has been previously sequenced for comparison.
3. Overlay with mineral oil (if necessary).
4. PCR conditions are 95°C for 4 min initial denaturation followed by 35 cycles of 95°C for 30 s, X°C for 30 s (*see Table 6* for specific annealing temperatures), 72°C for 30 s. This is followed by a single synthesis step of 72°C for 5 min.
5. Check the efficiency of PCR by electrophoresis of 5  $\mu$ L of PCR product on a 1% agarose gel.

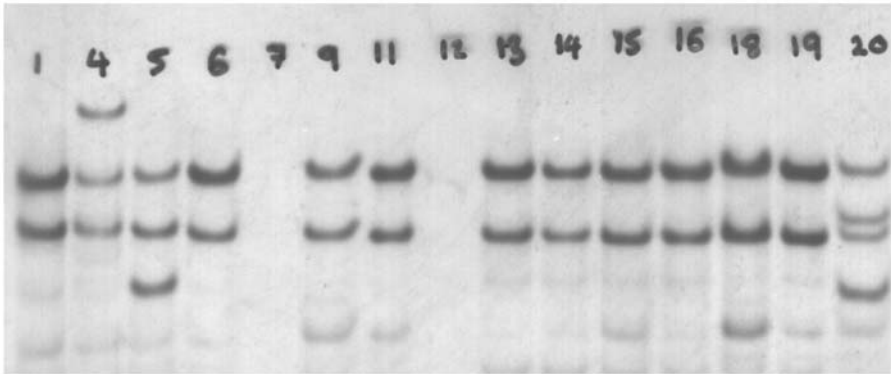


Fig. 3. SSCP analysis of exon 8 of APC. Variants are seen in lanes 4, 5, and 20.

### 3.4.2. Polyacrylamide Gel Analysis

1. Prepare a 15% non-denaturing polyacrylamide gel.
2. Mix 10  $\mu\text{L}$  of PCR product with 5- $\mu\text{L}$  of loading buffer and load onto gel.
3. Electrophorese the gel at 10 V/cm for 2 h.
4. Stain gel with ethidium bromide.
5. The presence of a small fragment as well as the normal-sized DNA band indicates the presence of a deletion (*see Note 12*).

## 3.5. Sequencing Analysis

### 3.5.1. PCR Conditions

The final concentration of components is: 1X Taq buffer, 2 mM magnesium chloride, 200  $\mu\text{M}$  dNTP mix, 1  $\mu\text{M}$  of each primer, 2 U of Taq polymerase, and 500  $\mu\text{g}$  of DNA in a 25- $\mu\text{L}$  reaction (*see Note 13*).

1. Prepare a master mix with all components except DNA. Aliquot 24  $\mu\text{L}$  into each PCR tube.
2. Add 1  $\mu\text{L}$  of DNA or 1  $\mu\text{L}$  distilled water for blank control into each tube.
3. Overlay with mineral oil (if necessary).
4. PCR conditions are 95°C for 4 min initial denaturation followed by 35 cycles of 95°C for 60 s, X°C for 60 s (*see Tables 3 and 5* for annealing temperatures), 72°C for 60 s. This is followed by a single synthesis step of 72°C for 5 min.
5. Check the efficiency of PCR by electrophoresis of 5  $\mu\text{L}$  of PCR product on a 1% agarose gel (*see Note 14*).
6. PCR fragments can be stored at 4°C or -20°C until ready to sequence.

### 3.5.2. Clean Up of PCR Product

A number of methods are available to clean up the PCR product to be sequenced, in order to remove any unincorporated primers. The steps listed here give reliable results on a routine basis.

1. Transfer 10  $\mu\text{L}$  of PCR product to a fresh tube.
2. Add 2  $\mu\text{L}$  of exonuclease 1 and 2  $\mu\text{L}$  of shrimp alkaline phosphatase.
3. Incubate at 37°C for 15 min.
4. Inactivate enzymes by incubation at 80°C for 15 min.

### 3.5.3. Cycle Sequencing

A number of kits are available for sequencing, and details of their use are supplied by the manufacturer. Further details are also given in Chapter 5. The method chosen will be dependent on normal procedures within the laboratory. All sequencing should be carried out in forward and reverse directions (*see Note 15*).

## 3.6. Linked and Intragenic Marker Analysis

There are four intragenic markers that can be used. The C to T restriction fragment-length polymorphism (RFLP) at nucleotide 1458 is identified following RsaI digestion of exon 11. The RFLP in exon 15 segment M is identified following MspI digestion. The G to T RFLP in the 5' untranslated region (UTR) is identified following DdeI digestion. An RFLP in the 3' UTR is identified following SspI digestion. There are six markers linked to APC that can be used (**Table 7**; **Note 16**).

### 3.6.1. PCR Conditions—Intragenic Markers

The final concentration of components is: 1X Taq buffer, 2 mM magnesium chloride, 200  $\mu\text{M}$  dNTP mix, 1  $\mu\text{M}$  of each primer, 2 U of Taq polymerase, and 500  $\mu\text{g}$  of DNA in a 25- $\mu\text{L}$  reaction (*see Note 17*).

1. Prepare a master mix with all components except DNA. Aliquot 24  $\mu\text{L}$  into each PCR tube.
2. Add 1  $\mu\text{L}$  of DNA or 1  $\mu\text{L}$  distilled water for blank control into each tube.
3. Overlay with mineral oil (if necessary).
4. PCR conditions are 95°C for 4 min initial denaturation followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. This is followed by a single synthesis step of 72°C for 5 min.

### 3.6.2. Restriction Digest and Electrophoresis for Intragenic Markers

1. Digest 15  $\mu\text{L}$  PCR product with 1  $\mu\text{L}$  of the appropriate restriction enzyme in a 25  $\mu\text{L}$  reaction for a minimum of 4 h.

2. Analyze 10  $\mu\text{L}$  of the digest on a 2% agarose gel containing ethidium bromide to resolve the alleles.

### 3.6.3. PCR Conditions—Linked Markers

The final concentration of components is: 1X Taq buffer, 2 mM magnesium chloride, 200  $\mu\text{M}$  dNTP mix, 400 nM of each primer, 2 U of Taq polymerase and 500  $\mu\text{g}$  of DNA in a 25- $\mu\text{L}$  reaction.

1. Prepare a master mix with all components except DNA. Aliquot 24  $\mu\text{L}$  into each PCR tube.
2. Add 1  $\mu\text{L}$  of DNA or 1  $\mu\text{L}$  distilled water for blank control into each tube.
3. Overlay with mineral oil.
4. PCR conditions are 95°C for 4 min initial denaturation followed by 22 cycles of 95°C for 30 s, X°C for 30 s (*see Table 7* for annealing temperatures), 72°C for 30 s. This is followed by a single synthesis step of 72°C for 5 min (*see Note 18*).

### 3.6.4. Linked Marker Analysis

The method of analysis will be dependent on the methods normally used within the user's laboratory.

1. For fluorescent analysis on an ABI373/377/310/3100 sequencer, mix 1  $\mu\text{L}$  of PCR product with 1.5  $\mu\text{L}$  of loading buffer, 3  $\mu\text{L}$  of formamide and 0.5  $\mu\text{L}$  of appropriate size standard and load 5  $\mu\text{L}$  of the denatured sample onto the gel and run under normal conditions. A 3-h run will usually separate the alleles sufficiently.
2. For non-fluorescent methods, *see Note 2*.

## 4. Notes

1. Some laboratories still use manual sequencing. This method is perfectly valid, and many standard protocols are available.
2. In addition, all the markers used can be analyzed by polyacrylamide gel/silver staining methods. The Model SA 32 system (Invitrogen) can be used to run denaturing gels if non-fluorescent analysis is carried out. Acrylamide for denaturing gels is a 40% ready-mixed solution at a 19:1 acrylamide/*bis* ratio. Gels can be silver stained, as described in **Subheadings 2.** and **3.** for SSCP gels.
3. All PCR assays should include a negative control (water blank), a normal DNA control, and a positive control (DNA with a known truncating mutation in the segment under analysis). If the analysis is for a presymptomatic test, the positive control should be a member of the family who carries the mutation.
4. Prior to sequencing, the region of exon 15 containing the mutation can be localized initially by DGGE/SSCP analysis to save sequencing of the entire segment amplified in the PTT reaction. Alternatively, once a panel of known truncating mutations is available, it is possible to run these on the PTT gel alongside the uncharacterized sample to localize the region in which the mutation lies.

5. One missense mutation in the APC gene, I1307K, can be detected by PTT. This truncation is an *in vitro* phenomenon and is believed to be caused by transcriptional or translational pausing or misincorporation during the reaction. This may be particularly ascribed to the nature of the mutation, which is a T to A transversion resulting in an extended poly A tract (A<sub>n</sub>). It is important to recognize this mutation, as it is not a cause of FAP, although it may increase the risk of the individual developing colorectal cancer (21).
6. All PCR assays should include a negative control (water blank), a normal DNA control, and a positive control (DNA with a known mutation in the exon or fragment under analysis). If the test is a presymptomatic test, the positive control should be a member of the family carrying the mutation. For SSCP or DGGE analysis of exons containing known polymorphisms (primarily exons 11 and 13 of APC), it is helpful to run homozygous and heterozygous controls so that patterns representing other variants can be distinguished. Exons 11 and 13 both contain di-allelic polymorphisms that are easily detectable by either method. Mutations in *cis* with these polymorphisms can be detected, but complex patterns are sometimes observed.
7. DGGE is a sensitive technique, but it cannot guarantee detection of all mutations. Several mutations that are not detected by this system using the conditions described here are in exon 7 (S254X; 779C > T) and exon 8 (R302X; 967C > T). If DGGE analysis is carried out and no mutations are detected, it is worth re-analyzing these exons by an alternative method such as SSCP. In addition, exon 3 is analyzed by DGGE in three fragments but can be analyzed by SSCP in a single assay with good resolution.
8. It is possible to analyze simultaneously in a multiplex PCR reaction to speed up analysis. Exons 3, 6, 8, and 13 can be analyzed together, as can 4, 7, 9, and 11, as well as 5, 10, 12, and 14.
9. Ideally, gels should be run at 4°C. If a cold room is not available, the SA32 system fits into cold cabinets supplied by Sanyo (Loughborough, Leicestershire, UK). The alternative to running the gels at 4°C is to add 10% glycerol to the gels and run them at room temperature.
10. These conditions are designed so that double-stranded DNA (dsDNA) remains on the gel, but single-stranded molecules are separated and can be resolved, allowing both SSCP and HA to be performed simultaneously. This is useful, because SSCP tends to be better at detecting point mutations and HA appears to be better at detecting frameshifts. Both types of mutations occur in APC.
11. Gels can be left in the fix solution overnight or even over the weekend if required, with no loss in resolution.
12. Both sets of primers used in this analysis are located on either side of the common 5-bp deletions at codon 1061 and 1309, respectively. However any 5-bp deletion within the fragment will yield an identical pattern, so it is still necessary to sequence any mutation identified this way to confirm the exact mutation present. There is at least one such 5-bp deletion located at codon 1055, which can be detected with the primers described for detection of the 1061 mutation.



13. When sequencing a novel variant, normal DNA should also be analyzed for comparison. If sequencing a case for presymptomatic diagnosis, both normal DNA and a sample from an individual known to carry the mutation should be analyzed.
14. It is important to obtain a good yield of PCR product, and to avoid a nonspecific amplification that would result in poor-quality sequencing results.
15. Commercially available cycle sequencing kits can often be diluted for use. 1–2  $\mu$ L of big dye kits (Amersham Pharmacia Biotech) can be used, and will yield a reliable sequence.
16. Markers D5S82 and D5S346 flank APC and are the most informative closest markers, and should be used as the first test. If used with a fluorescent analysis system, these can be run together in a duplex reaction. Equally, D5S346, MBC, D5S122, and D5S299 can be analyzed together in a multiplex reaction if they are fluorescently labeled.
17. A water blank, DNA samples that are homozygous for alleles 1 and 2, and a sample known to be heterozygous should be run as controls.
18. If linked markers are to be run on a non-fluorescent system imaged by silver staining, 30 cycles of PCR will be needed.

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## Multiple Endocrine Neoplasia Types 1 and 2

Sian Ellard

### 1. Introduction

Multiple endocrine neoplasia (MEN) is characterized by the occurrence of tumors involving two or more endocrine glands within a single patient. Multiple endocrine neoplasia types 1 (MEN1) and 2 (MEN2) are autosomal dominant cancer syndromes caused by mutations in the *MEN1* (1) and *RET* (2) genes, respectively.

#### 1.1. MEN Type 1

MEN1 is characterized by tumors of the parathyroids, pancreas, and anterior pituitary (*see ref. 3*). Familial MEN1 is defined as the presence of tumors in two of these three organs with a first-degree relative who has one or more of these tumors. Mutations in the *MEN1* gene (located on chromosome 11q13) are scattered throughout the gene, and the majority of these (>80%) are inactivating, consistent with those expected in a tumor-suppressor gene.

#### 1.2. MEN Type 2

MEN2 is characterized by medullary thyroid carcinoma (MTC), pheochromocytomas, and parathyroid tumors. Three distinct clinical subtypes have been described, which are caused by constitutively activating mutations of the *RET* proto-oncogene (4). Loss-of-function mutations scattered throughout the gene cause Hirschsprung's disease, a congenital abnormality characterized by the absence of parasympathetic ganglia from the myenteric plexuses of the terminal hind gut (5).

##### 1.2.1. MEN Type 2A

MEN2A describes the association of MTC with pheochromocytomas (in 50% of patients) and parathyroid tumors (20% of patients). Approximately

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85% of MEN2A patients have a mutation in codon 634 of the *RET* proto-oncogene (located on chromosome 10q11.2). Mutations of five other cysteine residues in exon 10 (codons 609, 611, 618, and 620) and 11 (codon 630) have also been described in MEN2A patients.

### 1.2.2. MEN Type 2B

MEN2B is characterized by the occurrence of MTC and pheochromocytoma in association with a Marfanoid habitus and mucosal neuromas. Parathyroid involvement is rare, and >95% of patients have an identical mutation of the *RET* proto-oncogene, M918T, in codon 16 (6). A second mutation, A883F (in exon 15), has been described in two unrelated patients (7).

### 1.2.3. Familial MTC

Familial MTC (FMTC) is a variant in which MTC is the sole manifestation of the syndrome. Mutations have been reported in codons 609, 611, 618, 620, and 634 (as for MEN2A), but are more frequently found in codons 609, 611, 618, and 620 (60% of patients) and less frequently in codon 634 (30% of patients) compared to MEN2A patients. Mutations in exons 13 and 14 (E768D, L790F, Y791F, and V804M) have also been reported in FMTC (8,9).

## 1.3. Utility of Diagnostic Molecular Genetic Testing

Molecular genetic testing by mutation screening of the *MEN1* and *RET* genes is used to establish a diagnosis. If a mutation is found, testing of family members who are at risk of inheriting the disorder is possible (10). Individuals without the mutation require no further follow-up. *MEN1* gene-mutation carriers have a high risk of developing MEN1—100% penetrance at age 60 (11)—and annual follow-up is recommended. Thyroidectomy during childhood is suggested for *RET* gene-mutation carriers with screening for pheochromocytoma (MEN2A and MEN2B) and parathyroid tumors (MEN2A) if appropriate. Since approximately 7% of sporadic MTC patients have a germline *RET* gene mutation (12–17), mutation analysis of apparently isolated cases of MTC is indicated (18).

## 2. Strategy

### 2.1. Restriction Digests for Detecting *RET* Mutations in Codon 634 (found in MEN2A and FMTC) and the M918T Mutation Detected in Most MEN2B Patients

It is possible to test for mutations of codon 634 (exon 11) of the *RET* gene by polymerase chain reaction (PCR) using the primers shown in **Table 1** (19) followed by digestion with specific restriction endonucleases (REs). The primers contain mismatches that create control restriction sites for the enzymes

**Table 1**  
**Sequences of Primers for Detection of *RET* Gene Mutations by PCR/Restriction Digest<sup>a</sup>**

Exon	Product, bp	Annealing temperature °C	Forward primer, 5'–3'	Reverse primer, 5'–3'
11	168	56	CATGAGGCCGAGCATACTCAGCC	CAGACAGCAGCGCCGAGACGATG

<sup>a</sup>As described by McMahon et al. (19).

**Table 2**  
**Detection of Codon 634 *RET* Mutations by PCR/Restriction Digest**

Codon 634 mutations	Enzyme	Estimated band sizes in basepairs, normal sequence	Extra bands created by mutation
TGC > TNN	Fnu4HI	104, 32, 14, 9, 3	118
TGC > CGC	HinPI	157, 11	101, 56
TGC > GGC	HaeIII	161, 7	95, 66
TGC > AGC	DdeI	152, 16	81, 71

DdeI, HaeIII, and HinPI/HhaI. Mutations of codon 634 create additional sites for these enzymes (see **Table 2**). The normal product contains four restriction sites for Fnu4HI, and one is lost in patients with certain codon 634 mutations (see **Table 2**).

The M918T mutation found in MEN2B patients may be detected by a restriction digest of an exon 16 PCR product (for primers, see **Table 3**) using FokI (6).

## 2.2. Mutation Screening of the *MEN1* and *RET* Genes

The coding region of the *MEN1* gene (exons 2–10) and exons 10, 11, and 13–16 of the *RET* gene are screened according to the phenotype. Sequencing is the author's preferred approach because it is the most sensitive method currently available, and once a mutation is found, no further characterization is required. However, it is more labor-intensive and expensive compared to other techniques such as denaturing high-performance liquid chromatography (DHPLC) and single-strand conformation polymorphism (SSCP) that other laboratories may prefer to adopt. The PCR products generated using the primers described in this chapter (see **Tables 3** and **4**) may be used for mutation screening by any of these other approaches.

## 2.3. Linkage for *MEN1* Families Without an *MEN1* Gene Mutation

Between 5% and 20% of *MEN1* patients may not harbor mutations in the coding region of the *MEN1* gene (1,11,20–22). These patients may have mutations in the promoter or untranslated regions. If the family structure is suitable and samples are available from relevant family members, gene tracking using microsatellite markers for the 11q13 region may be useful because no second locus for *MEN1* has been reported to date. Primer sequences for a set of microsatellite markers are shown in **Table 5**.

## 3. Materials

Analytical-grade reagents should be used at all stages, unless otherwise indicated.

**Table 3**  
**Sequences of Primers for Detection of *RET* Gene Mutations by PCR/Sequencing**

Exon	Product, bp	Annealing temperature °C	Forward primer, 5'–3'	Reverse primer, 5'–3'
10	187	65	GCG CCC CAG GAG GCT GAG TG <sup>a</sup>	CGT GGT GGT CCC GGC CGC C <sup>a</sup>
11	416	58	CAT GAG GCC GAG CAT ACT CAG CC <sup>b</sup>	CCT CGT CTG CCC AGC GTT G <sup>a</sup>
13	239	58	CTC TCT GTC TGA ACT TGG GC <sup>a</sup>	TCA CCC TGC AGC TGG CCT TA <sup>a</sup>
14	328	65	AAG ACC CAA GCT GCC TGA C <sup>a</sup>	GTG GTG GGT CAG GGT GTG G <sup>a</sup>
15	234	58	GAC TCG TGC TAT TTT TCC TC <sup>a</sup>	TAT CTT TCC TAG GCT TCC CA <sup>a</sup>
16	192	58	AGG GAT AGG GCC TGG GCT TC <sup>a</sup>	TAA CCT CCA CCC CAA GAG AG <sup>a</sup>

<sup>a</sup>As described by Ceccherini et al. (23).

<sup>b</sup>As described by McMahon et al. (19).

**Table 4**  
**Sequences of Primers for Detection of *MEN1* Gene Mutations by PCR/Sequencing**

Exon	Product, bp	Annealing temperature °C	Forward primer, 5'–3'	Reverse primer, 5'–3'
2A	463	58	GGA ACC TTA GCG GAC CCT GGG AG <sup>a</sup>	GAG ACC TTC TTC ACC AGC TCA CGG <sup>a</sup>
2B	630	65	ATC GAC GAC GTG GTG CGC CTG TTT G <sup>a</sup>	GGG TCA TGG ATA AGA TTC CCA CC <sup>b</sup>
3	376	58	CAG AGG ACC CTC TTT CAT TAC CTC C <sup>a</sup>	GGC TCT TCT GTC TTC CCT TCC TAT G <sup>c</sup>
4	191	58	GGG CCA TCA TGA GAC ATA ATG <sup>a</sup>	CTG CCC CAT TGG CTC AG <sup>c</sup>
5/6	297	65	CCT GTT CCG TGG CTC ATA ACT C <sup>c</sup>	CTC AGC CAC TGT TAG GGT CTC C <sup>c</sup>
7	251	58	GGC TGC CTC CCT GAG GAT C <sup>c</sup>	CTG GAC GAG GGT GGT TGG <sup>c</sup>
8	218	65	GTG AGA CCC CTT CAG ACC CTA C <sup>c</sup>	TGG GAG GCT GGA CAC AGG <sup>c</sup>
9	246	65	GGG TGA GTA AGA GAC TGA TCT GTG C <sup>c</sup>	TGT AGT GCC CAG ACC TCT GTG <sup>c</sup>
10	551	58	CGG CAA CCT TGC TCT CAC C <sup>c</sup>	CCC ACA AGC GGT CCG AAG TCC <sup>c</sup>

<sup>a</sup>As described by Chandrasekharappa et al. (1).

<sup>b</sup>Ellard, unpublished.

<sup>c</sup>As described by Lemmens et al. (21).



**Table 5**  
**Sequences of Primers for *MEN1* Microsatellite Markers**

Microsatellite marker <sup>a</sup>	Annealing temperature °C	Fluorescent label	Forward primer, 5'–3'	Reverse primer, 5'–3'
D11S480 <sup>b</sup>	56	FAM	CCC TCT TGC CTG TGT TGA AAT	TTT GAG GTA GGC TTC GTA TA
D11S1883	56	FAM	TTC AGT AAC AGG AGA CAA AAG G	TGG TTT CGG ATC TCT TCT CA
PYGM <sup>b</sup>	56	FAM	CTA GCA GAG TCC ACC TAC TG	GCT GTC AGG TAG CAA CTG AC
D11S449 <sup>c</sup>	56	TET	GGT GAA AAA ACA CAC TTG TCT G	GGC GAC ATA GTG AGA TCC TGT
D11S4155	56	TET	CCC GAG ATG CTG GTG AG	GCC TTT GAG ATG GAA GCC
D11S1889	65	TET	AGC TGG ACT CTC ACA GAA TG	CAA GAG GCT GGT AGA AGG TG
D11S913	58	HEX	CAT TTG GGA AAT CCA GAA GA	TAG GTG TCT TAT TTT TTG TTG CTT C

<sup>a</sup>Markers D11S480–PYGM are proximal and D11S449–D11S913 are distal to the *MEN1* gene.

<sup>b</sup>As described by Larsson et al. (24).

<sup>c</sup>As described by Courseaux et al. (25).

### 3.1. PCR

1. *Taq* polymerase: The author uses Amplitaq Gold (5 U/ $\mu$ L) supplied with Buffer II (without magnesium chloride) and 25 mM magnesium chloride from Applied Biosystems (ABI) (Warrington, UK). Store at  $-20^{\circ}\text{C}$ .
2. Deoxynucleotide triphosphates (dNTPs): The author uses a 20-mM mix from AbGene (Epsom, UK) with each dNTP at 5 mM. Store at  $-20^{\circ}\text{C}$ . The modified mix for use in conjunction with deazaGTP is prepared from 100-mM individual stocks (AbGene) diluted to 5 mM deoxythymidine triphosphate, deoxycytidine triphosphate, deoxyadenosine triphosphate (dTTP, dCTP, dATP) and 3.8 mM (dGTP).
3. Deaza-7-dGTP 10 mM (Roche, Lewes, UK). Store at  $-20^{\circ}\text{C}$  (*see Note 1*).
4. Oligonucleotide primers: Diluted to 5  $\mu$ M from primers synthesized commercially by MWG (Milton Keynes, UK) and purified by reverse-phase HPLC. Store at  $-20^{\circ}\text{C}$ .
5. Dimethyl sulfoxide (DMSO) (Sigma, Poole, UK). Store aliquots at  $-20^{\circ}\text{C}$ .
6. Betaine 5 M (Sigma). Store at  $4^{\circ}\text{C}$  (*see Note 1*).
7. Agarose (Helena Bioscience, Sunderland, UK).
8. 5X TBE electrophoresis buffer (Sigma).
9. Gel-loading buffer (Sigma).

### 3.2. Restriction Digest and Analysis

1. Restriction enzymes from commercial sources.
2. Acrylamide: Use 29:1 acrylamide/*bis*-acrylamide mix. The author uses a 30% ready-mixed solution (BioRad, Hemel Hempstead, UK).
3. Ammonium persulfate (AMPS) (Sigma). Prepare a 10% solution and store aliquots at  $-20^{\circ}\text{C}$ .
4. *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Sigma).
5. Ethidium bromide solution: Dilute 10 mg/mL stock (Sigma) in 1X TBE to a final concentration of 0.5  $\mu$ g/mL.

### 3.3. Sequencing Analysis

1. QIAquick PCR product purification kit (individual columns for purification using centrifugation or 8-strip columns for purification using a vacuum manifold (Qiagen, Crawley, UK).
2. BigDye Terminator Sequencing Kit (ABI).
3. BigDye dilution buffer (ABI).
4. Oligonucleotide primers: Diluted to 0.8  $\mu$ M from primers synthesized commercially by MWG and purified by reverse-phase HPLC. Store at  $-20^{\circ}\text{C}$ .
5. 96% ethanol (Merck, Lutterworth, UK).
6. 3 M sodium acetate (Sigma).
7. Qiagen DyeEx 96 kit (Qiagen).
8. Quick-Comb<sup>TM</sup>-96 (Sigma).
9. Acrylamide: Use 19:1 acrylamide/*bis*-acrylamide mix. The author uses a 40% ready-mixed solution (Biorad).

10. AMPS (Sigma). Prepare a 10% solution and store aliquots at  $-20^{\circ}\text{C}$ .
11. TEMED (Sigma).
12. Urea (Anachem, Luton, UK).
13. Mixed-bed resin (Sigma).
14. 5X TBE electrophoresis buffer (Sigma).
15. High-Dye formamide (ABI). This high-grade formamide is recommended by ABI.
16. Gel-loading buffer (ABI).

### 3.4. Linkage Analysis

1. Genescan TAMRA-500 size standards (ABI).
2. Acrylamide: Use 19:1 acrylamide/*bis*-acrylamide mix. The author uses a 40% ready-mixed solution (Biorad).
3. AMPS (Sigma): Prepare a 10% solution and store aliquots at  $-20^{\circ}\text{C}$ .
4. TEMED (Sigma).
5. Urea (Anachem).
6. Mixed-bed resin (Sigma).
7. 5X TBE electrophoresis buffer (Sigma).
8. High-dye formamide (ABI).
9. Gel-loading buffer (ABI).

## 4. Methods

### 4.1. PCR

1. Prepare a master mix according to **Table 6**. Aliquot into thin-walled 0.6- or 0.2-mL PCR tubes.
2. Add 2.5  $\mu\text{L}$  (or 10  $\mu\text{L}$  for RET codon 634 analysis) DNA (20 ng/ $\mu\text{L}$ ). Predictive tests are performed on duplicate samples (*see* **Notes 2** and **3**).
3. Place on a thermal cycler with the hot lid preheated.
4. PCR cycling conditions: Initial denaturation  $95^{\circ}\text{C}$  12 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 1 min,  $56^{\circ}\text{C}$ ,  $58^{\circ}\text{C}$ , or  $65^{\circ}\text{C}$  (*see* **Tables 1–4**) for 1 min,  $72^{\circ}\text{C}$  for 1 min, with a final extension at  $72^{\circ}\text{C}$  for 10 min.
5. Check PCR products by adding 2  $\mu\text{L}$  gel-loading buffer (Sigma) to 5  $\mu\text{L}$  product and loading on a 2% agarose gel. Run gel for 20 min at 120 V with ethidium bromide (0.5  $\mu\text{g}/\text{mL}$ ) in both the gel and TBE running buffer.
6. Visualize on a UV transilluminator and record the gel image.

### 4.2. Restriction Digest and Analysis

1. Add 1  $\mu\text{L}$  of enzyme, 2  $\mu\text{L}$  of restriction enzyme buffer, and 2  $\mu\text{L}$  water to a 15- $\mu\text{L}$  aliquot of the PCR product. Incubate for at least 3 h at  $37^{\circ}\text{C}$ .
2. Carry out electrophoresis of RET exon 16 Fok I products on an agarose gel as described in **Subheading 3.1.5**.
3. Prepare a 13% polyacrylamide gel (29:1).
4. Run RET exon 11 codon 634 products for 3 h at 100 V.

**Table 6**  
**PCR Master Mixes: Reagent Volumes/Sample**

Reagent ( $\mu\text{L}$ )	RET codon 634	RET exons 10, 11, 13, 14, 15, and 16	MEN1 exons 2A, 2B, 3, 4, 5/6, 7, 8, 9, and 10	MEN1 microsatellite markers
Water	51	15.4	6.8	15.4
10X PCR	10	2.5	2.5	2.5
25 mM $\text{MgCl}_2$	4	1.5	2.5	1.5
dNTP mix (5 mM each)	4	1	–	1
dNTP mix (3.8 mM dGTP)		–	1	–
DeazaGTP		–	1.25	–
DMSO		–	1.25	–
Betaine		–	5	–
Forward primer	10	1	1	1
Reverse primer	10	1	1	1
Amplitaq Gold	1	0.1	0.2	0.1
Total volume	90	22.5	22.5	22.5

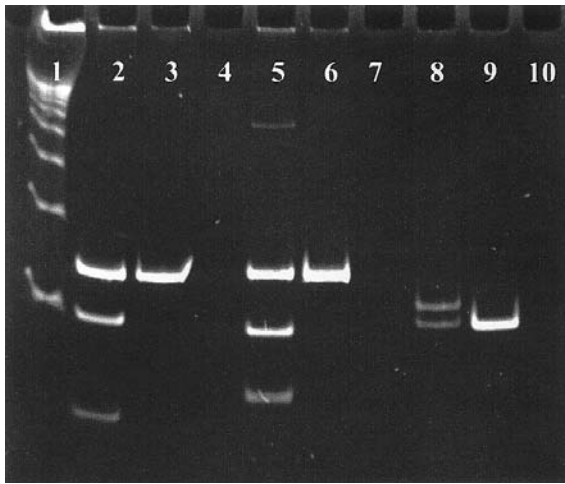


Fig. 1. Detection of codon 634 *RET* gene mutations by PCR/restriction digest. Lane 1 contains a 100-bp size standard. Lanes 2–4 show normal, positive (C634R), and negative samples digested with *HinPI*. Lanes 5–7 show positive (C634G), normal, and negative samples digested with *HaeIII*. Lanes 8–10 show positive (C634Y), normal, and negative samples digested with *Fnu4HI*.

5. Stain the gel with ethidium bromide (0.5  $\mu\text{g}/\text{mL}$  in 1X TBE) for 15 min.
6. Visualize on a UV transilluminator and record the gel image (**Fig. 1**).
7. Interpret the results according to **Table 2**.

### 4.3. Sequencing Analysis

The protocol described here refers to the use of an ABI377 DNA sequencer (ABI) but these methods are generally applicable to other commercially available instruments—for example, the ALF Express or MegaBACE (Amersham Pharmacia, Little Chalfont, UK) and LI-COR analyzer (MWG).

1. Purify PCR products in order to remove residual primers by using QIAquick columns according to the manufacturer's instructions. These columns may be purchased individually or in strips of 8 for higher throughput. See **Note 4** for an alternative method.
2. Prepare forward and reverse sequencing reactions using the appropriate primers listed in **Tables 3** and **4** by adding 1  $\mu\text{L}$  Big Dye Dilution buffer, 2  $\mu\text{L}$  Big Dye Terminator Sequencing Mix, 2  $\mu\text{L}$  primer (at 0.8  $\mu\text{M}$ ) to 5  $\mu\text{L}$  purified PCR product in a 0.2-mL thin-walled PCR tube or a 96-well plate.
3. Sequencing reaction conditions: 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min.

**Table 7**  
**Amino Acid Polymorphisms in the *MEN1* and *RET* Genes**

Gene	Exon	Nomenclature	Nucleotide change
<i>MEN1</i>	2	V50V	GTC > GTA
<i>MEN1</i>	2	L143L	CTC > CTT
<i>MEN1</i>	2	S145S	AGC > AGT
<i>MEN1</i>	3	R171Q	CGG > CAG
<i>MEN1</i>	4	L256L	CTT > CTC
<i>MEN1</i>	9	L432L	CTG > CTA
<i>MEN1</i>	9	D418D	GAC > GAT
<i>MEN1</i>	10	A541T	GCA > ACA
<i>RET</i>	11	G691S	GGT > AGT
<i>RET</i>	13	L769L	CTT > CTG
<i>RET</i>	14	S836S	AGC > AGT
<i>RET</i>	14	P841P	CCG > CCT
<i>RET</i>	15	S904S	TCC > TCG

- Excess fluorescent dye terminators are removed from reactions in individual tubes by ethanol-precipitation. Add 10  $\mu$ L water, 2  $\mu$ L sodium acetate and 50  $\mu$ L 96% ethanol to the sequencing reaction. Transfer the mix to a 0.5-mL microfuge tube and incubate at room temperature for 15–30 min. Centrifuge at 17,530g for 15 min. Remove the supernatant using a vacuum pump, and wash the pellet (which is probably not visible) with 200  $\mu$ L 70% ethanol. Centrifuge at 17,530g rpm for 2 min and remove the supernatant as before. Air-dry for 10–20 min. Alternatively, if the sequencing reactions were performed in a 96-well plate use the DyeEx 96 kit for 96-well plates according to the manufacturer's instructions.
- Prepare the sequencing gel for the ABI377 (36-cm plates) by mixing 18 g urea, 22.5 mL ultra pure distilled water, 5.3 mL 40% acrylamide and 0.5 g mixed-bed resin (Sigma). Stir to dissolve for 5–10 min. Place 10 mL 5X TBE in the lower chamber of the filter apparatus. Filter the gel solution and allow to degas for 1–2 min. Add 250  $\mu$ L 10% ammonium persulfate solution (APS) and 35  $\mu$ L TEMED. Swirl gently to mix. Allow the gel to polymerize for 2 h before use.
- Add 3  $\mu$ L of loading buffer (5:1 formamide:loading buffer) to the sequencing reaction pellets and denature at 95°C for 2 min. Place on ice prior to loading 1.5  $\mu$ L (48-well gels) or 0.5  $\mu$ L (96-well gels—see **Note 5**).
- Run the gel for 7 h using the appropriate run module for the sequencing kit (Seq Run 36E-1200 for the BigDye Terminator kit).
- Check the gel tracking, and using the Sample Manager program, analyze sequences defining start and end points in order to maximize signal-to-noise ratios.
- Use the FACTURA software to assign IUB (International Union of Biochemists) codes to sequences.

10. Analyze the data using the Sequence Navigator program to compare patient sequences with normal control sequence by creating “shadows” to identify ambiguities (see **Note 6**).
11. If a variant is detected, check that it is not one of the polymorphisms listed in **Table 7**. Novel missense mutations in the *MEN1* gene should be treated with caution if other family members require predictive tests (see **Note 7**).

#### 4.4. Linkage Analysis

1. Dilute PCR products in water (1/10 for FAM or TET-labeled products, 1/5 for HEX-labeled products) and pool the products by color/size as appropriate.
2. Add 1  $\mu\text{L}$  of diluted/pooled PCR product to 2  $\mu\text{L}$  loading buffer (5:1:1 formamide:loading buffer:GS500 size standard) and denature at 95°C for 2 min. Place on ice prior to loading 1.5  $\mu\text{L}$  per well (48-well gel).
3. Run the gel for 2.5 h using the appropriate Genescan run module (GS Run 36C–2400).
4. Check the gel tracking and analyze the products using Genescan software.
5. Identify alleles using Genotyper software.
6. Construct haplotypes according to the least number of recombinations.
7. Calculate LOD scores using the linkage analysis program M-LINK (available at <http://linkage.rockefeller.edu/software/linkage>) if required.

#### 5. Notes

1. Betaine, DMSO, and deaza-GTP are used to enhance the PCR amplification of CG-rich sequences present in exons 2 and 10 of the *MEN1* gene that can be recalcitrant to amplification.
2. The policy of the author’s laboratory for predictive tests is to extract DNA from duplicate blood samples and test both DNA samples.
3. A positive control DNA sample (ideally from a family member) should always be included when performing a predictive test in addition to a negative control (water instead of DNA). If PCR product is evident in the negative control, then the test is invalid and must be repeated using fresh reagents.
4. A cheaper alternative to using spin columns to purify PCR products is digestion of the products with two units each of shrimp alkaline phosphatase (Amersham Pharmacia) and Exonuclease 1 (Amersham Pharmacia) for 1.5 h at 37°C.
5. Samples may be loaded onto 96-well sequencing gels using a multichannel loader. However, we prefer to add samples individually onto a dry-load comb that is then inserted into the gel.
6. The Sequence Navigator software is used to identify heterozygous mutations. The patient sample is compared to a normal control by creating shadows for both the forward and reverse sequences. The presence of a mutation or polymorphism is indicated by asterisks for the forward and reverse shadows at the same position within the sequence. Examples are shown in **Figs. 2** and **3**.
7. The possibility that a novel missense mutation in the *MEN1* gene is a rare polymorphism should not be discounted. Testing of other family members (parents and

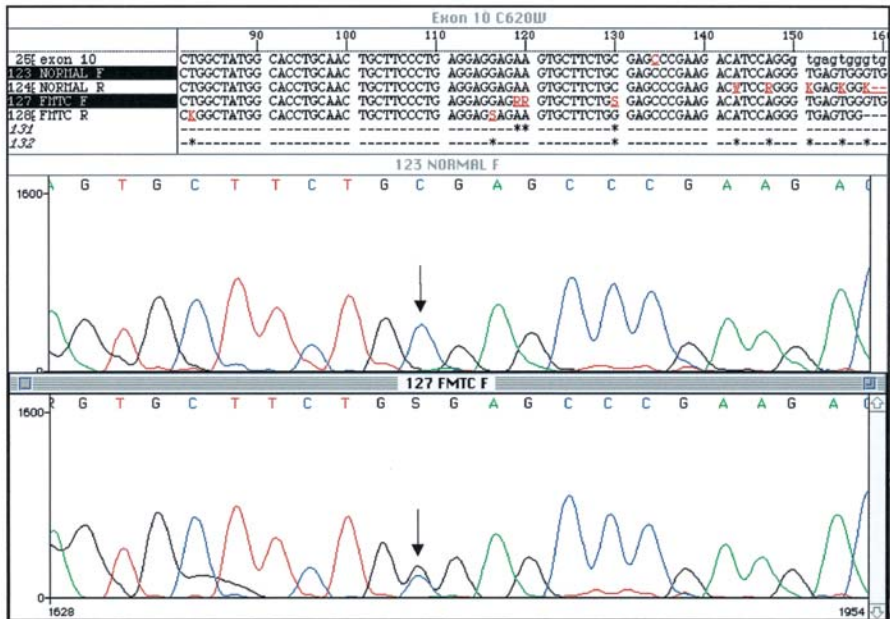


Fig. 2. Detection of C620W mutation by sequencing exon 10 of the *RET* gene in a patient with FMTc. Part of the sequencing navigator layout is shown, together with electropherograms for a control (upper panel) and patient (lower panel).

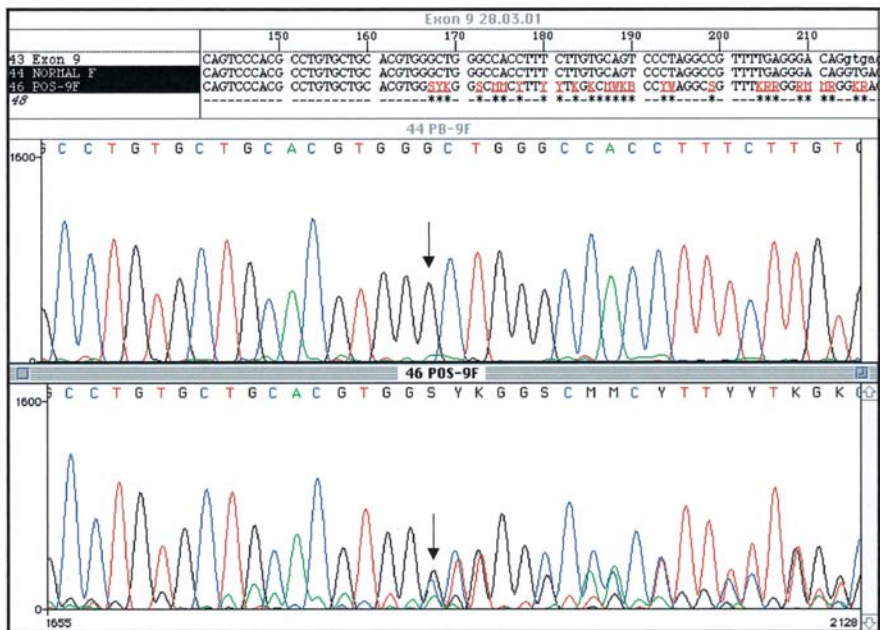


Fig. 3. Detection of 1414delG *MEN1* mutation by sequencing. The control sequence is shown in the upper panel and the patient sequence in the lower panel.



siblings) is recommended in order to demonstrate co-segregation of the variant with the disease. Alternatively, testing of the parents may establish that the mutation is *de novo*. If the amino acid residue is conserved between species or a different mutation at the same codon has been reported previously, this would increase the likelihood of a pathogenic mutation. However, if at-risk relatives seek predictive testing, this should be discussed during counseling.

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## Neurofibromatosis Type 1

### *A Common Familial Cancer Syndrome*

**Meena Upadhyaya, Peter Thompson, Song Han,  
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#### **1. Introduction**

Neurofibromatosis type 1 (NF1) is one of the most common dominantly inherited neurogenetic disorders, affecting about 1 in every 4000 individuals worldwide. It is fully penetrant by the age of five. The condition is characterized by multiple café-au-lait spots, benign neurofibromas, and Lisch nodules (1). Other clinical manifestations include abnormalities of the cardiovascular, gastrointestinal, renal, and endocrine systems; major orthopedic problems; facial and body disfigurement; and cognitive deficit and malignancy, with tumors of the peripheral nerve sheath and central nervous system (CNS). About one-quarter of NF1 patients develop one or more of these clinical complications, demonstrating the significant morbidity and mortality associated with this disorder. However, the clinical expression varies greatly from one patient to another, between families, and even within a given family carrying the same underlying gene lesion. The establishment of diagnostic criteria for NF1 (2) has potentiated the clinical diagnosis of NF1 in most patients. Neurofibromas are a hallmark feature of NF1. Each neurofibroma is composed of Schwann cells (60%–80%), fibroblasts, perineurial cells, axons, and mast cells. The cellular sources of the various neurofibroma-associated mitogens, the occurrence of specific receptors for each of these factors, and their functional role in tumor development remain unclear. Indeed, the characterization of novel tumor growth-promoting factors and their receptors in both benign and malignant cancers may help to define potential treatment strategies for NF1.

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Children with NF1 have an increased risk of developing myeloid disease, particularly juvenile myeloid leukemia and myelodysplastic syndrome. In practice, the overall frequency of cancers in NF1 patients is about twice that observed in the general population. Currently, about 22,000 individuals in UK are affected by NF1 and because there is still no treatment, this presents a major health burden to the community.

### **1.1. The NF1 Gene as Tumor-Suppressor**

There is strong evidence that *NF1* gene is a tumor-suppressor, a gene whose normal function is to inhibit or control cell division. The Knudson “two-hit” tumor-suppressor model predicts that all somatic cells of an affected person harbor a constitutional (germline) *NF1* mutation. The occurrence of a second *NF1* mutation involving the normal allele is then predicted to result in the loss of cell-cycle control, leading to tumor formation (3–5). The observation of multiple distinct *NF1* somatic mutations in neurofibromas from the same NF1 patients (6,7) reveals that each second hit is an independent event. This in turn suggests that the somatic mutational spectrum of the *NF1* gene may be an important contributor to the variable clinical expression of NF1.

### **1.2. NF1 Gene**

The *NF1* gene located at 17q11.2 spans 350 kb of genomic DNA, contains 60 exons and encodes a 12 kb mRNA transcript (8). Three of the exons are alternatively spliced. The stop codon lies in exon 49, which also includes a 3′ untranslated region (3′ UTR). Neurofibromin, the *NF1* gene product, is ubiquitously expressed, and exhibits structural and sequence similarity to an evolutionarily conserved family of proteins, the mammalian GTPase-activating protein (GAP)-related proteins. The most highly conserved region of the protein is the NF1 GAP-related domain (GRD), encoded by exons 21–27a. To date, this is the only domain of neurofibromin to which a function has been ascribed. Activated p21-ras (ras with bound GTP) can transform fibroblasts and increases cell proliferation in vitro. Neurofibromin has been shown to downregulate p21-ras (ras-GDP). Thus, inactivation of neurofibromin has been theorized to be involved directly in tumorigenesis.

Within the *NF1* gene, the large intron 27b contains three embedded genes (*OMGP*, *EVI2B*, and *EVI2A*) that are transcribed in the opposite orientation to the *NF1* gene. Each of the three embedded genes comprises two exons. *EVI2A*, a small gene encoding a 232-amino acid polypeptide, is expressed in the brain and the bone marrow; sequence analysis suggests that it has a transmembrane domain (9). *EVI2B* is also a small gene that encodes a 448-amino-acid protein with unknown function, and it is expressed exclusively in the bone marrow (10). *OMGP* encodes a 416-amino acid cell-adhesion molecule, and is expressed pri-

marily in oligodendrocytes (*11*). It is unknown whether embedded genes play a role in the expression of *NF1*.

Fluorescence *in situ* hybridization (FISH) analysis and genomic sequence homology of amplified polymerase chain reaction (PCR) products have revealed that there are numerous *NF1* homologous loci spread across the genome (*12,13*).

The *NF1* promoter is located in a CpG island and exhibits a high degree of sequence conservation with other species (*14*). The 3' UTR of the *NF1* gene extends 3.5 kb downstream of the translation stop signal in exon 49 (*15*).

The application of reverse transcriptase-PCR (RT-PCR) reveals *NF1* mRNA in almost all tissues. Indeed, lymphocytes express enough *NF1* mRNA to provide a ready source of RNA for mutational analysis. Three alternatively spliced isoforms with significantly different expression patterns have been identified.

### 1.3. *NF1* Gene Mutations

The mutation rate for *NF1* is 1 in 10,000 gametes/cell/generation, approx 10-fold higher than that found for most other inherited disease genes. The germline mutational spectrum of the *NF1* gene has been well-characterized (*16*), with new mutations occurring in about one-half of all *NF1* individuals. To date, some 440 *NF1* gene lesions have been logged in either the Human Gene Mutation Database (<http://www.hgmd.org>) or the NNF (National Neurofibromatosis Foundation) Mutation Database (<http://www.nf.org/nf1>). However, no clustering of mutations within the *NF1* gene is apparent. Among germline mutations, 80% are truncating, and approx 30% involve abnormalities of splicing (*6,16–18*). By contrast, data on the tumor-associated somatic mutations of this gene are still sparse.

Characterizing mutations in the *NF1* gene has presented a considerable challenge because of the combination of the size of the gene, the absence of any obvious mutational clustering, and the wide diversity of mutations (*16*). The existence of numerous *NF1* pseudogenes adds to the complexity of PCR-based mutation analysis (*12,13,19*). FISH has been used to identify very large gene deletions or rearrangements that include the *NF1* gene (*20,21*). Pulsed-field gel electrophoresis has been used to identify gross deletions and insertions within the *NF1* gene (*22–24*). Conventional Southern blot analysis has been employed for the study of medium-size gene rearrangements (*24–26*).

Single-strand conformational polymorphism (SSCP) (*24,25,27,28*) and heteroduplex analysis (HA) often have been used in the past to detect microlesions (*25,29,30*). Other methods—including denaturing gradient gel electrophoresis (DGGE) (*31*), temperature gradient gel electrophoresis (*17*), the protein truncation test (PTT) (*3,17,26,32–34*), the long reverse transcriptase-polymerase chain reaction (LRT-PCR) (*35*), and direct sequencing (*17*) have all been used for mutation detection; no single technique has been completely effective.

#### **1.4. Mutations in the Non-Coding Regions of the *NF1* Gene**

The 3' untranslated region (UTR) of the human *NF1* gene is 3.5 kb in length and highly conserved during evolution (36), indicating that this region may be important for mRNA stability or translational efficiency. Although there may be mutations in the 3' UTR that are of pathological significance, only one 3' UTR mutation has been reported to date (37,38).

Although the *NF1* gene-promoter sequence is highly conserved evolutionarily (14), no pathological mutations in this region have yet been detected (39). Their eventual identification will help to define the mechanisms that regulate neurofibromin expression.

#### **1.5. Molecular Testing**

NF1 is usually diagnosed on the basis of clinical and ophthalmic examination, especially in those patients who are more than 5 yr of age. However, in many pediatric situations, a sufficiently precise clinical diagnosis may not be possible, and these families may be greatly aided by the identification of mutations. Some clinicians feel that it is important to make diagnosis early so that patients can be offered counseling and guidance, and that children can be monitored for such complications as optic glioma and hypertension. Since pre-emptive prophylactic clinical intervention is not yet possible, it is difficult to justify presymptomatic testing. Population and newborn screening are not yet available for NF1 worldwide. Inherited mutations in the *NF1* gene have been identified in some families who do not meet the NIH diagnostic criteria for NF1; these include those with segmental NF1, Watson syndrome, or familial café-au-lait spots only (40,41). Detection of *NF1*-specific mutations in such families would help to confirm the clinical diagnosis.

Although the demand for prenatal diagnosis is not high, it is occasionally sought. A low demand for prenatal testing could be a result of the inability of this test to predict the severity of the condition. Prenatal diagnosis can be performed by direct gene analysis if a specific mutation has already been identified in the family. Alternatively, prenatal diagnosis can be based on genetic linkage if samples are available from enough key family members (42–44). The identification of specific mutations will permit molecular diagnosis independent of family structure, and will be particularly helpful for large numbers of isolated cases.

Fetal DNA specimens may be obtained by amniocentesis or chorionic villus sampling (CVS). The recent application of high-throughput mutation detection techniques has substantially improved the *NF1* mutation detection efficiency and may have an impact on molecular testing for this condition.



## 1.6. Current Molecular Methodology

The authors currently use both FISH and DHPLC (denaturing high-performance liquid chromatography) for *NF1* molecular diagnosis, and have found these methodologies to be both efficient and highly complementary. A significant minority (5%) of patients with *NF1* are heterozygous for a germline 1.5-Mb deletion at 17q11.2, which includes the *NF1* gene (45). The deletion is associated with a typical phenotype of minor facial abnormalities, mental retardation, learning disabilities, and early or excessive burden of cutaneous or plexiform neurofibromas (46). At present, the most effective method to detect large deletions is FISH performed on standard metaphase spreads using probes that are specific for the *NF1* gene region. If the patient does not have a deletion of the relevant probe, a signal will be seen on both chromosome 17 homologs at 17q11.2. A single signal in each case examined is indicative of a chromosomal deletion (Fig. 1).

## 1.7. Overall Approach

### 1.7.1. FISH

In the authors' laboratory, two probes have proven to be reliable: P1-9, which is 65 kb in size and maps to exons 2 to 11, and P1-12, which is 55 kb in size and maps to intron 27b. These were kindly supplied by Dr. Bruce Korf (21). An indirectly labeled hapten-conjugated nucleotide such as biotin-16-dUTP is then incorporated into the DNA by nick translation. This is then detected via a fluorescein isothiocyanate (FITC)-labeled avidin. For best results with FISH, labeled probe fragments of approx 200–500 basepairs (bp) are required. Longer sequences result in decreased probe penetration and increased nonspecific binding (leading to a higher background signal).

### 1.7.2. DHPLC

DHPLC is a modification of the basic gel-based heteroduplex mutation analysis method (47), and is applied to those samples without a FISH-detectable deletion. This methodology relies upon the rapid separation and visualization of homo- and heteroduplex DNA molecules using an ion-pair reverse-phase liquid chromatography system and has proven to be both rapid and sensitive. The sensitivity of the analysis is maximized by maintaining the liquid chromatography column at a temperature that favors partial strand denaturation in the presence of bp mismatching. Preliminary local assessment of the DHPLC system has successfully identified every lesion within exon 16 of the *NF1* gene (48). Subsequently, DHPLC conditions were further optimized to screen the entire coding region of the *NF1* gene; the sensitivity of this approach for *NF1*

mutational analysis is now 95% (Han et al., 2001) confirming DHPLC to be a rapid and efficient tool for *NF1* mutational analysis (30).

The authors carry out DHPLC on a WAVE™ DNA fragment analysis system using a DNASep® column (Transgenomic, Inc., Omaha, NE 68107) (48). DNASep® columns contain non-porous alkylated polystyrene-divinylbenzene particles that are both electrically neutral and hydrophobic. The successful resolution of heteroduplexes from homoduplexes requires an elution gradient at a partially denaturing temperature. At this temperature, only heteroduplexes are destabilized by the mismatched bases, and are therefore slightly more melted than the homoduplexes, resulting in earlier elution than the homoduplexes. This special resolution temperature ( $T_r$ ) can be predicted by use of the DHPLCMelt software (<http://insertion.stanford.edu/melt.html>).

The DHPLCMelt software successfully determined the optimal  $T_r$  for each of the 60 fragments containing individual exons of the *NF1* gene. Because of the unavailability of complete sequence for intron 1 of the *NF1* gene in GenBank, empirical determination of the  $T_r$  and elution gradient of the intron 1 fragment was performed (48).

## 2. Materials

### 2.1. FISH

#### 2.1.1. Nick Translation

1. Nick translation system. The authors use the Invitrogen (Paisley, Scotland) nick translation system. Detailed instructions for use are supplied with the kit. Store at  $-20^{\circ}\text{C}$ .
2. Indirectly labeled hapten. The authors use biotin-16-dUTP (Boehringer, Mannheim, Germany) for detection using avidin and biotinylated anti-avidin. Digoxigenin-11-dUTP (Boehringer Mannheim) can be used as an alternative, with detection via fluorescein-labeled antibodies. Store at  $4^{\circ}\text{C}$ .
3. 1% agarose check gel. Add 1 g agarose (Roche) to 100 mL TAE working solution, dissolve, and add 5  $\mu\text{L}$  ethidium bromide working solution ( $10\text{ mg mL}^{-1}$ ). To make a stock solution of TAE buffer, add 48.4 g Tris-HCl (USB), 17 mL glacial acetic acid (BDH, Poole, UK), and 3.72 g EDTA (Sigma, St. Louis, MO) to deionized water and make up to 1 L. Store at room temperature. Dilute 1 : 10 with deionized water to make a working solution.
4. Ethidium bromide (Sigma Aldrich, Steinheim, Germany). Add 1 mL sterile water to 10 mg ethidium bromide to make the working solution. Store at  $4^{\circ}\text{C}$ .
5. Bromophenol blue (Sigma).
6. 1-kb ladder (Invitrogen).

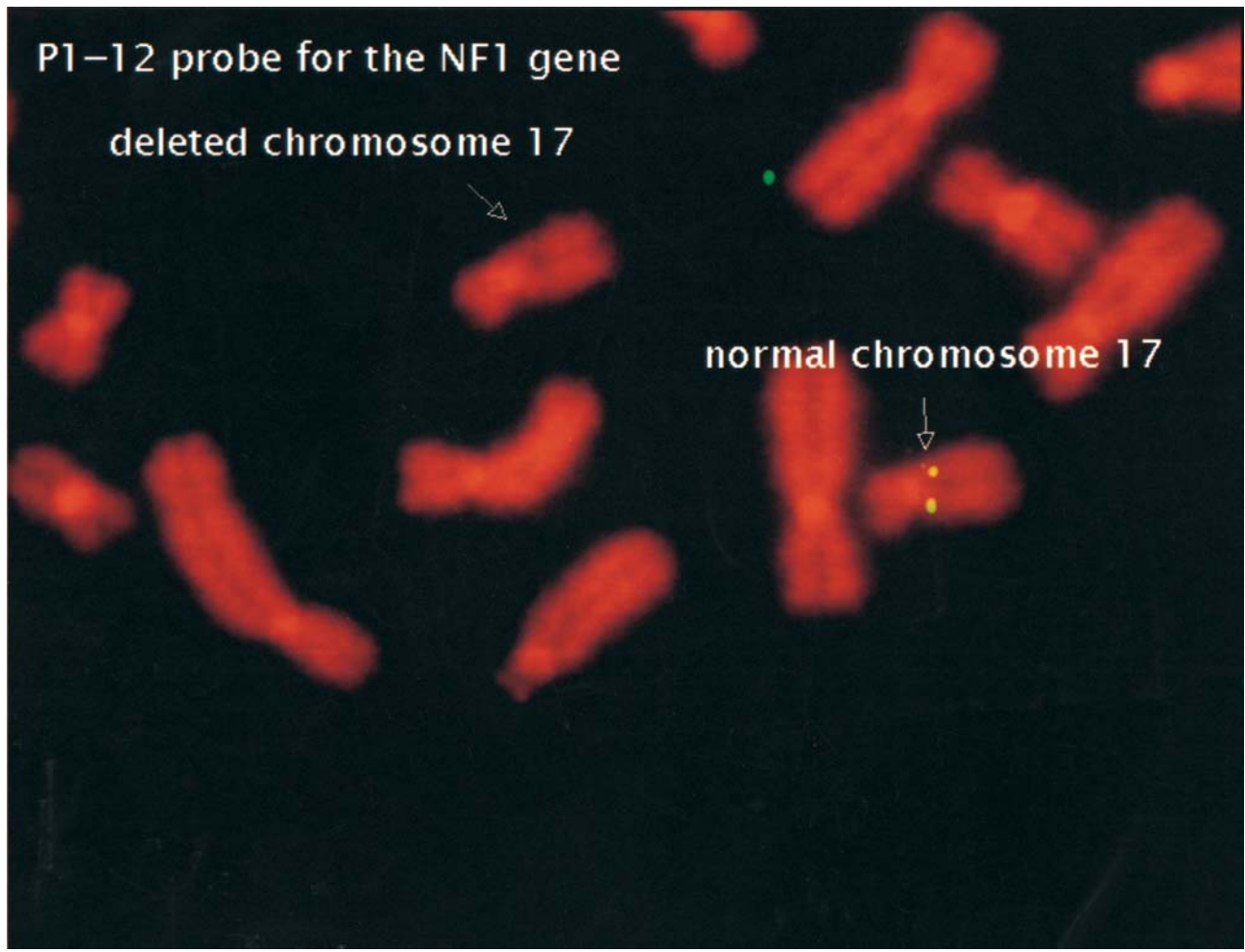
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Fig. 1. (see facing page) FISH studies showing a deletion of the *NF1* gene.

P1-12 probe for the NF1 gene

deleted chromosome 17

normal chromosome 17



### 2.1.2. Probe Preparation

1. Cot-1 DNA (InVitrogen).
2. 3 M sodium acetate. Add 110.8 g sodium acetate (Sigma) to sterile water and make up to 100 mL. Store at room temperature.
3. Hybridization buffer. The hybridization buffer contains 50% formamide, 10% dextran sulfate, and 2X standard saline citrate (SSC). Dissolve 20% dextran sulfate (Sigma) in molecular-grade formamide (BDH, Poole, UK). Aliquot into microcentrifuge tubes and keep as a stock solution at  $-20^{\circ}\text{C}$ . To prepare a working solution, add 600  $\mu\text{L}$  sterile water and 150  $\mu\text{L}$  20X SSC to 750  $\mu\text{L}$  stock solution. Store at  $-20^{\circ}\text{C}$ .
4. 20X SSC. Add 175.3 g sodium chloride (Sigma) and 88.22 g sodium citrate (Sigma) to sterile water and make up to 1 L. Mix to ensure that all of the chemicals have dissolved. Store at room temperature.
5. Ethanol (BDH).

### 2.1.3. Hybridization and Probe Detection

1. Denaturation solution. The denaturation solution consists of 70% formamide (BDH) in 2X SSC at pH 7.0–8.0. Prepare by adding 350 mL formamide to 50 mL 20X SSC and 100 mL sterile water; adjust pH to 7.0–8.0 using HCl. The denaturation solution can be used for up to 1 wk. Store at room temperature.
2. Ethanol wash solutions. 500 mL w/v solutions of 70%, 90%, and 100% are prepared using 100% ethanol and sterile water. The ethanol wash solutions can be used for up to 1 wk. Store at room temperature.
3. 2X SSC posthybridization wash solution. Prepare by adding 50 mL 20X SSC to 450 mL sterile water, and adjusting the pH to 7.0–7.5 with 1 M NaOH. The wash solution can be used for up to 1 wk.
4. 4T buffer. Add 100 mL 20X SSC and 250  $\mu\text{L}$  Tween-20 (Sigma) to 400 mL sterile water. Make up fresh buffer for each hybridization.
5. Blocking solution. Add 250 mg Boehringer blocking agent (Boehringer Mannheim) to 50 mL 4X SSC to make a 0.5% working solution. Store in 2.5-mL aliquots in glass bottles at  $-20^{\circ}\text{C}$ . Use a new aliquot for each hybridization.
6. Fluorescein-avidin. To make a working solution, add 1  $\mu\text{L}$  stock fluorescein-avidin solution, as supplied by the manufacturer (Vector Laboratories, Burlingame, CA), to 100  $\mu\text{L}$  blocking solution. Make sufficient for each day's hybridization (120  $\mu\text{L}$  per slide). Keep at  $4^{\circ}\text{C}$  while in use. Discard any excess at the end of the day.
7. Biotinylated anti-avidin. To make a working solution, add 1  $\mu\text{L}$  stock biotinylated anti-avidin solution (Vector Laboratories) to 100  $\mu\text{L}$  blocking solution. Make sufficient for each day's hybridization (60  $\mu\text{L}$  per slide). Keep at  $4^{\circ}\text{C}$  while in use. Discard any excess at the end of the day. The stock solution is prepared by resuspending the powder in 1 mL sterile water.
8. DAPI and propidium iodide (PI) counterstain. Prepare by adding 5  $\mu\text{L}$  DAPI (Sigma), and 5  $\mu\text{L}$  PI (Sigma) working solution (0.5 mg/mL) to 1 mL of Vectashield mountant (Vector Laboratories). To make the working solution, add 1  $\mu\text{L}$  5 mg/mL PI stock solution to 9  $\mu\text{L}$  sterile water.

## 2.2. DHPLC

1. 2 M triethylammonium acetate (TEAA): (cat. no. 553303, Transgenomic) store at 4°C.
2. Acetonitrile (ACN): HPLC-grade, (cat. no. 152857L, Merck), store at room temperature, is toxic and should be used in a chemical fume hood. Wear gloves when handling this solution.
3. 0.5 M EDTA: cat. no. 306, Teknova. Store at room temperature.
4. Milli-Q water or HPLC-grade water.
5. DNASep™ column: store at room temperature.
6. Size standard: this standard consists of a restriction digest of plasmid pUC18 by *Hae*III and represents a pool of nine DNA fragments with the following sizes 80, 102, 174, 257, 267, 298, 434, 458, 587 bp. The DNA digest standard is used to evaluate instrument performance for size-based DNA fragment separations.
7. Mutation standard: this standard consists of a combination of two analogous 209-bp fragments representing A and G alleles at position 168 of the polymorphic *DYS271* locus. Upon heating and renaturation, this fragment mixture forms two homoduplexes and two heteroduplexes that are used to check instrument parameters for heteroduplex-based mutation screening.
8. A thermal cycler with a heated lid.

## 3. Methods

### 3.1. FISH

#### 3.1.1. Nick Translation

During the nick translation procedure, all solutions are kept on ice.

1. To a 1.5-mL microcentrifuge tube, add the following; 5 µL nucleotide mix without deoxythymidine triphosphate (dTTP), X µL template DNA (a total amount of 1 µg probe DNA is required), 2.5 µL biotin-16-dUTP, make up to 45 µL with Y µL of distilled water.
2. Mix gently, then add 5 µL DNA polymerase I/DNase I solution. Mix gently but thoroughly, and pulse-spin.
3. Incubate at 16°C until the DNA is fragmented to the appropriate size. For probes P1-9 and P1-12, this will take between 90 and 120 min.
4. Place the solution on ice and run a check gel to ensure that the DNA fragments are 200–500 bp in size.
5. Add 5 µL reaction to 3 µL bromophenol blue and load on the 1% agarose gel.
6. Add 5 µL of the 1-kb ladder to a separate well. Carry out the electrophoresis at 100 V for 30 min and examine the gel on a UV trans-illuminator. If the fragments are too large, incubate for a longer period of time. If the fragments are too small, discard the mixture and start again.
7. When the fragments are of the appropriate size, halt the reaction by adding 5 µL stop solution.

### 3.1.2. Probe Preparation

It is advisable to test a single aliquot of probe for its hybridization efficiency before using the probe in a diagnostic context. If the hybridization is successful, all of the DNA may be prepared for use.

1. To a microcentrifuge tube, add the following; 4  $\mu\text{L}$  probe solution from the nick translation reaction (this should contain approx 60 ng DNA), 2.5  $\mu\text{L}$  Cot-1 DNA, 0.5  $\mu\text{L}$  3 M sodium acetate, and 64  $\mu\text{L}$  of ethanol.
2. Mix well and leave at  $-20^{\circ}\text{C}$  for 30 min.
3. Centrifuge at 17,000g for 10 min.
4. Remove the supernatant, being careful not to disturb the deposit that should be visible at the bottom of the tube.
5. Dry off the pellet in a  $37^{\circ}\text{C}$  incubator. This should take approx 30–60 min, with occasional gentle mixing by hand. If there are problems in resuspending the mixture, a pipet may be used to mix the solution. The probe is now ready for use.

### 3.1.3. Hybridization and Probe Detection

#### 3.1.3.1. SLIDE DENATURATION

Slides are made by standard cytogenetic laboratory procedures. For best results, make the slides up 2–3 h prior to use. Old or very fresh slides may be used, but they yield poorer hybridization and chromosome morphology, respectively.

1. Place the slides in a Coplin jar containing 50 mL of denaturation solution in a  $73^{\circ}\text{C}$  water bath inside a Class 1 cabinet for 2 min. It is not recommended that more than four slides are used at one time.
2. Transfer the slides to 70% ice-cold ethanol for 2 min, followed by 2 min in 90% and 100% ethanol at room temperature.
3. Remove the slides, drain the excess ethanol by touching the bottom of the slide on tissue, and wipe the back of the slide dry with a tissue. Allow to air-dry in an upright position. This usually takes 3–5 min.

#### 3.1.3.2. PROBE DENATURATION

10  $\mu\text{L}$  probe mixture per slide is generally used.

1. Place the probe mixture in the  $73^{\circ}\text{C}$  water bath for 10 min.
2. Place the probe in the  $37^{\circ}\text{C}$  water bath for approx 60 min.

#### 3.1.3.3. HYBRIDIZATION

1. Apply the 10- $\mu\text{L}$  probe mixture to an appropriate area of the slide.
2. Immediately place a 22 mm  $\times$  22 mm cover slip over the probe solution and allow the probe solution to spread evenly under the cover slip. Any air bubbles are removed by applying gentle pressure with a pipet tip.

3. Seal with rubber solution.
4. Place the slides in a humidified chamber and allow to hybridize overnight in a 37°C incubator.

#### 3.1.3.4. POSTHYBRIDIZATION WASHES

1. Remove the rubber solution and then gently remove the cover slip.
2. Immediately place the slides in a Coplin jar containing 50 mL of 2X SSC wash solution in a 73°C water bath inside a Class 1 cabinet for 2 min. Do not process more than four slides at once.
3. Wash in 4T buffer in a Coplin jar for 1 min at room temperature.
4. Add 60 µL blocking solution, pre-warmed to 37°C, to the slide, apply a 64 mm × 22 mm cover slip and incubate in the humidified chamber at 37°C for 10 min.

#### 3.1.3.5. DETECTION OF PROBE SIGNAL

1. After incubation in blocking solution, gently remove the cover slip. Add 60 µL avidin-FITC working solution to the slide, seal with a cover slip and incubate in the humidified chamber at 37°C for 15 min.
2. Gently remove the cover slip and wash 3 × 2 min in 4T buffer in Coplin jars at room temperature.
3. Add 60 µL biotinylated anti-avidin working solution to the slide, seal with a cover slip, and incubate in a humidified chamber at 37°C for 15 min.
4. Gently remove the cover slip and wash 3 × 2 min in 4T buffer as previously.
5. Repeat **steps 1 to 2**.
6. Drain the excess 4T buffer by touching the bottom of the slide on tissue and wipe the back of the slide dry with a tissue.
7. Add 30 µL DAPI/PI counterstain to slides and apply a 64 mm × 22 mm cover slip. The slides are now ready for analysis. Slides can be stored at 4°C prior to analysis.

#### 3.1.4. Visualization of the Probe Signal

For FISH analysis, a fluorescence microscope with a 50-W (or preferably a 100-W) high-pressure mercury lamp and the appropriate filter set (FITC/PI/DAPI) is needed.

In the authors' laboratory, 30 cells are checked for the deletion.

## 3.2. DHPLC

### 3.2.1. Preparation of Buffers

Use clean glass bottles for the preparation and storage of buffers. Always rinse flasks and bottles three times before buffers are prepared.



### 3.2.1.1. BUFFER A

0.1 M triethylammonium acetate (TEAA), 1 mM EDTA, pH 7.0. Use 50-mL and 1000-mL volumetric flasks to prepare this buffer. Measure out 50 mL 2 M TEAA, and transfer to a 1000-mL volumetric flask. Rinse the 50-mL volumetric flask 3 times with HPLC-grade water and transfer the rinse to the 1000-mL volumetric flask to ensure proper volume is transferred. Add 200  $\mu$ L 0.5 M EDTA into the 1000-mL flask. Adjust the final vol to 1000 mL with HPLC-grade water.

### 3.2.1.2. BUFFER B

0.1 M TEAA, 25% ACN, pH 7.0. Prepare buffer B using the 1000-mL, 250-mL, and 50-mL volumetric flasks. Measure 50 mL 2 M TEAA and 250 mL ACN and transfer to a 1000-mL volumetric flask. Rinse each three times to ensure all the TEAA/ACN is transferred. Bring the volume up to approx 950 mL with HPLC-grade water. Seal the flask to prevent evaporation, and leave at room temperature for at least 10 min. Adjust the final volume to exactly 1000 mL.

### 3.2.1.3. BUFFER C

75% ACN, pH 7.0. Using a 250-mL volumetric flask, transfer 750 mL of acetonitrile to a 1000-mL flask, add 250 mL HPLC-grade water to bring the final vol to 1000 mL.

### 3.2.1.4. BUFFER D

8% ACN, pH 7.0. Prepare this by using a glass graduated-cylinder to measure 80 mL HPLC-grade ACN, transfer into a 1000-mL volumetric flask, and bring the final vol to 1000 mL with HPLC-grade water. This solution is used to rinse the needle and injection valve. It is also used to deliver sample through the injection valve without any losses.

## 3.2.2. PCR Amplification of 60 Exons of NF1 Gene

Primers and PCR conditions for all 60 exons of the *NF1* gene are listed in **Table 1**. However, the guidelines for the design of new primers are available from Transgenomic™ (application note No. 101). For PCR amplification, a thermal cycler with a heated lid should be used. The use of mineral oil for the prevention of evaporation of PCR reaction is not recommended.

Heteroduplexes are formed by subjecting amplified PCR product to 95°C for 5 min and allowing it to cool to 25°C at a ramp of 0.02°C per s. This step can be added to a PCR regimen after the final extension.

*(Text continues on page 300)*



Table 1

## NF1 Primer Sequences and DHPLC Fragments Analysis

Exon	Oligo sequence (5'-3')	Boundaries	Fragment Size (bp)	Conditions		Sources
				PCR Ta (°C)	DHPLC Tr (°C)	
1	CAGACCTCTCCTTGCCTCTT GGATGGAGGGTCGGAGGCTG		438	65	61	Purandare et al., 1995
2	AAGCTGTTAACGTGTTTTTTTTTTC AAGAAAAGAAAGCAAATCCCC	-37 +47	228	58	55	Abernathy et al., 1997
3	TTTCACTTTTCAGATGTGTGTTG TGGTCCACATCTGTACTTTG	-77 +84	245	60	54	Purandare et al., 1995
4a	TTAAATCTAGGTGGTGTGT AAACTCATTTCTCTGGAG	-130 +195	517	55	50,54, 59	Upadhyaya, et al., unpublished
4b	GATGATGTCTTGCTATGTTGC TTGGTGTCTAGTTCAGCAC	-159 +90	366	63	54,59	This report
4c	TTTCTTAGCAGACAACATATCGA AGGATGCTAACAAACAGCAAAT	-173 +67	308	58	54	This report
5	GAAGGAAGTTAGAAGTTGTGACA CAATCGTATCCTTACCAGCCAT	-81 +15	172	57	54	Upadhyaya, et al., unpublished
6	CATGTTTATCTTTTAAAAATCTTGCC ATAATGGAAATAATTTTGCCCTCC	-115 +28	301	59	51,56	Upadhyaya, et al., unpublished
7	ACATCTGGAATAGAAGAAACTTCA CAGTAACAACAAAAGCAAGTCC	-58 +145	377	64	52,57	This report
8	GGATTTTACTGCCATTTGTGTG TAACAGCATCAGTAAATATAGTTAGATA	-108 +45	276	62	52,57	Upadhyaya, et al., unpublished
9	TTGAAGTTCGTTTCAAGACC ACGCAAAGAAAAGAAAGAAAA	-137 +60	272	56	54	This report
9a	CTGTGGCTCAGAACACTAAAA CACATGCAGTGCTCATTAGA	-150 +124	308	63	55,60	This report
10a	ACGTAATTTTGTACTTTTCTTCC CAATAGAAAGGAGGTGAGATTC	-47 +43	222	58	53,58	Purandare et al., 1995
10b	ATTATCCTGAGTCTTACGTC TAACTTAGTGTGATAATTTGAGA	-47 +46	229	54	54	Upadhyaya, et al., unpublished
10c	ATTGAAGTTTCCTTTTTTCTTGC GTATAGACATAAACATACCATTTCC	-27 +134	275	57	54,59	This report
11	CCAAAAATGTTGAGTGAGTCT ACCATAAAACCTTTGGAAGTG	-55 +121	256	60	54	This report
12a	AAACCTTACAAGAAAATAAGCT ATTACCATTCCAATATCTTCCA	-130 +50	303	55	50,54	Purandare et al., 1995
12b	CTCTTGGTTGTCAGTGCTTC CAGAAAACAACAGAGCACAT	-50 +55	261	58	57	This report

Table 1 (continued)

Exon	Oligo sequence (5'-3')	Boundaries	Fragment Size (bp)	Conditions		Sources
				PCR Ta (°C)	DHPLC Tr (°C)	
13	GTCTTCCACCCTTGACTCTC GCTACTTGAATTTCCCCTGT	-22 +115	387	64	56,61	This report
14	GCTCTTCCTACTCCTTTTGG TTTCTGTTGCTAAGGGCATA	-96 +21	191	58	60	This report
15	ACTTGGCTGTAGCTGATTGA ACTTTACTGAGCGACTCTTGAA	-71 +92	247	65	56	This report
16	TGGATAAAGCATAAATTTGCAAGT TAGAGAAAGGTGAAAAATAAGAG	-75 +33	549	60	54,59	Li et al., 1995
17	TCTCTAGGGGGTCTGTCTTC CACCTAGTTTGTGTGCAGT	-64 +122	326	56	55	Purandare et al., 1995
18	AGAAGTTGTGTACGTTCTTTTCT CTCCTTTCTACCAATAACCGC	-147 +97	367	58	55	Purandare et al., 1995
19a	TCATGTCACTTAGGTTATCTGG TAAAACCCACTAATACTGAAGG	-120 +38	242	65	56	This report
19b	TGAGGGGAAGTGAAAAAAT GGCTTTATTTGCTTTTTGCT	-72 +47	236	56	53,58	This report
20	CCACCTGGCTGATTATCG TAATTTTTGCTTCTCTTACATGC	-148 +72	402	64	50,55,60	Purandare et al., 1995
21	TGGTCTCATGCACTCCATA CATCTTCTTCTGGCTCTGA	-140 +122	474	58	53,58	This report
22	TGCTACTCTTTAGCTTCTCTAC CCTTAAAAGAAGACAATCAGCC	-74 +95	331	58	58	Purandare et al., 1995
23-1	TTTGTATCATTCAITTTGTGTGTA AAAAACACGGTTCTATGTGAAAAG	-144 +34	282	57	56	Purandare et al., 1995
23-2	CTTAATGTCTGTATAAGAGTCTC ACTTTAGATTAATAATGGTAATCTC	-92 +40	268	58	53,58	Purandare et al., 1995
23a	AGCCAGAAATAGTATACATGATTGGGT CTATTTTCTGCCAGAATAGTAGA	-246 +137	446	62	56	Purandare et al., 1995
24	TTGAACCTTTTGTTTTCATGTCTT GGAATTTAAGATAGCTAGATTATC	-71 +36	266	58	53,58	Purandare et al., 1995
25	CCTGTTTTTATTGTGTAGATACTTCA TAAGTGGCAAGAAAATTACCA	-17 +19	134	53	56	Abernathy et al., 1997
26	AATCTAATGACTTTGCATTTTTG ATCTAAATTTAAACGGAGAGTG	-27 +52	226	60	52,57	Abernathy et al., 1997
27a	GTTACAAGTTAAAGAAATGTGTAG CTAACAAGTGGCCTGTGTGCAAAC	-74 +77	298	60	58	Purandare et al., 1995
27b	TTTATTTGTTTATCCAATTATAGACTT TCCTGTAAAGTCAACTGGGAAAAAC	-85 +100	296	53	50,54	Purandare et al., 1995

Table 1 (continued)

Exon	Oligo sequence (5'-3')	Boundaries	Fragment Size (bp)	Conditions		Sources
				PCR Ta (°C)	DHPLC Tr (°C)	
28	TTTCCTTAGGTTCAAAACTGG CTAGGGAGGCCAGGATATAG	-9 +75	517	64	54,59	This report
29	TCACCCCGTCAACCACACTTT GCAACAACCCCAAATCAAACCTGA	-25 +45	411	57	60	Upadhyaya, et al., unpublished
30	CAACTTCATTTGTGTTTTCTCCTAG CTTTGAATTCTCTTAGAATAATTGTTA	-25 +54	282	57	52,57	Abernathy et al., 1997
31	ATAATTGTTGATGTGATTTTCATTG AATTTTGAACCAGATGAAGAG	-58 +172	424	64	52,57	Upadhyaya, et al., unpublished
(32)	ATCTAGTATTTTTGAGGCCTCAG CAGATATGCTATAGTACAGAAGG	-130 +41	312	58	51,56	Abernathy et al., 1997
33	TCCTGCTTCTTACAGGTTATT AAGTAAATGGAGAAAGGAAGTGC	-16 +113	409	64	53,58	Abernathy et al., 1997
34	TTTTCTGTCTTACTTGTTCCTTTA CAGTCCATGCAAGTGTTTTT	-36 +133	384	57	53, 58	This report
35	GCATGGACTGTGTATTGGTA TGCAATTAAGATCCACAGA	-114 +143	319	65	51,56	This report
36	GTTCTGTGGATCTTTAAATTGC CATTGACCTCAAATTTAAACG	-24 +99	238	62	53,58	This report
37	CATTCCGAGATTCAGTTTAGGAG AAGTAACATTCAACTGATACCC	-75 +59	236	58	54	Abernathy et al., 1997
38	CTATGTCATGATTCATCTACTAGC CTAAATTTGAGTAATCTAGGAACCTC	-48 +44	233	60	54,59	Abernathy et al., 1997
39	CTACTGTGTGAACCTCATCAACC GTAAGACATAAGGGCTAACTTACTTC	-45 +112	284	62	51,56	Abernathy et al., 1997
40	TCAGGGAAGAAGACCTCAGGAGATGC TGAACCTTCTGTCTGCCACGCAACC	-82 +114	328	59	56	Abernathy et al., 1997
41	GTGCACATTTAACAGGTAATAT CTTCTAGGCCATCTCTAGAT	-112 +125	373	65	56	Previously designed
42	CTTGGAAGGAGCAAACGATGGTTG CAAAAACCTTGCTACACTGACATGG	-73 +125	356	60	50,55,60	Abernathy et al., 1997
43	TTTTCTTTTTAGTGTATCCCATT GATTCTAAGAAATGGCTGGAA	-74 +90	287	53	50,55	This report
44	CACGTTAATCCCTATCTTGC TGAGAAGTAGAAGACTGTATCC	-26 +121	268	64	53,58	Upadhyaya, et al., unpublished
45	CATGAATAGGATACAGTCTTCTAC CACATTACTGGGTAAGCATTTAAC	-97 +71	269	63	56	Abernathy et al., 1997
46	AAATGTTCTCTGTTGACTT CATCAACCATCCTTCTCCAGA	-34 +34	211	60	56	Upadhyaya et al., unpublished

**Table 1 (continued)**

Exon	Oligo sequence (5'-3')	Boundaries	Fragment Size (bp)	Conditions		Sources
				PCR Ta (°C)	DHPLC Tr (°C)	
47	CTGTTACAATTAAGATACCTTGC	-57	185	62	55	Abernathy et al., 1997
	TGTGTGTTCTTAAAGCAGGCATAC	+81				
48	TTTTGGCTTCAGATGGGGATTAC	-67	351	66	54,59	Abernathy et al., 1997
	AAGGGAATTCCTAATGTTGGTGTC	+67				
48a	ATTCCTTCTGAAAACCAAGG	-116	280	65	53,58	This report
	AAGGCAGACTGAGCTTACAG	+110				
49	CTGGGAGAAACAGGCTATAC	-212	363	62	60	This report
	AGCAAGCTTCACACGATCT	+11				

Ta, annealing temperature for PCR amplification; Tr, resolution temperature for DHPLC analysis.

### 3.2.3. Selection of Oven Temperature

The optimal melting temperature for the sequence of interest can readily be predicted by appropriate software if the sequence is known. Apart from WAVEMaker™ software, the Stanford University's DHPLC melt program (available from website <http://insertion.stanford.edu/melt.html>) is also widely employed. The majority of the sequence of the *NF1* gene is available, and can be retrieved from GenBank (Accession nos. AC004022 and AC004562). The complete sequence of intron 1 is still not available, and thus the conditions for amplification of fragment 1 of the *NF1* gene are empirical.

### 3.2.4. Temperature and Gradient Conditions

The temperature and gradient conditions for the analysis of an amplicon constitute the most important part of the procedure. Gradients for mutation detection comprise a DNA-loading step, the linear separation gradient, a clean-off step, and finally, equilibration. Once the method has been tested, it can be copied from one application folder to another, and thus used extensively for future analysis.

*NF1* exon 22 fragment is presented as an example. The resolution Temperature (Tr) is predicted by using Stanford University's DHPLC melt program. The melting temperature of the entire fragment and each portion of fragment is given, as well as gradient conditions corresponding to each temperature.

**Table 2**  
**Melt Gradient Condition**

Temperature (°C)	B1	B2	B3
58	50.0	53.4	58.8
57	50.0	54.8	60.2
56	50.0	56.2	61.6
55	50.0	57.6	63.0
54	50.0	58.9	64.3

where B = 0.1 M TEAA, pH 7.0, 25% ACN.

<u>Melting temperature (°C):</u>	<u>Zone of NF1 exon 22 (bases)</u>
54	1–12,325–331
55	13–37,323–324
56	38–64,212–322
57	65–166,208–211
58	167–207

This sequence should be run at all of the following temperatures to ensure detection of all polymorphisms.

Recommended temperature: 58°C.

The following concentrations are rough recommendations only. Some adjustment may be necessary. Run from B1 to B2 in 0.5 min, then B2 to B3 in 3.0 min.

For setting up a method on the WAVE™ instrument, the recommended temperature predicted from the Stanford melt program should be used.

The start gradient conditions (0.5 min) may be obtained from the percentage of buffer B given in column B3 of **Table 2** (58.8°C in the example shown). The Wave machine requires the operator to enter the concentration of buffer A, which may be obtained by subtracting the percentage of buffer B from 100%. In our laboratory, subtracting 2 from the starting percentage of buffer B was found to give a retention time of between 3 and 4 min for DNA fragments analyzed. The starting percentage of A in the example shown would be 43% (100–57). However, it may be necessary to optimize this according to the fragment size.

The gradient is formed so that the concentration of buffer B increased by 7% at 4 min. Thus, the value of the percentage of buffer A entered at 4 min would be 36% in the example shown. The flow rate was set to 0.9 mL.min<sup>-1</sup>.

**Table 3**  
**Sample Positioning Scheme for WAVE™ Auto Sampler Plate**

1	9	17	25	33	41	49	57	65	73	81	89
2	10	18	26	34	42	50	58	66	74	82	90
3	11	19	27	35	43	51	59	67	75	83	91
4	12	20	28	36	44	52	60	68	76	84	92
5	13	21	29	37	45	53	61	69	77	85	93
6	14	22	30	38	46	54	62	70	78	86	94
7	15	23	31	39	47	55	63	71	79	87	95
8	16	24	32	40	48	56	64	72	80	88	96

### 3.2.5. Setting Up Sample Table

Select the right method and the right position for the sample under analysis. The positioning scheme for the Auto Sampler plate is displayed in **Table 3**.

When placing samples in the Auto Sampler, make sure that it is in the right position. There is a cooling system in Auto Sampler to prevent evaporation of samples under higher temperatures. This cooling temperature must be adjusted according to environmental temperature. Heavy condensation may interfere with the system.

Click *Data Acquisition* icon; select the required sample table.

Click *Start Series* when the oven temperature is ready to start run.

Chromatograms for *NF1*-specific mutations are included in **Fig. 2**.

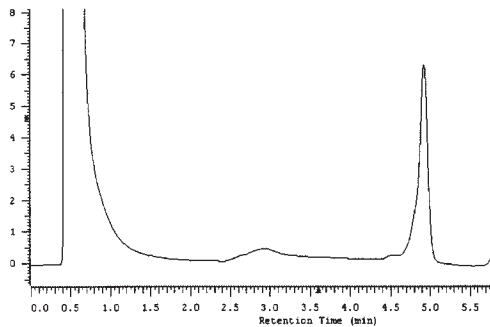
See also **Table 4** for PCR plate settings.

## 4. Notes

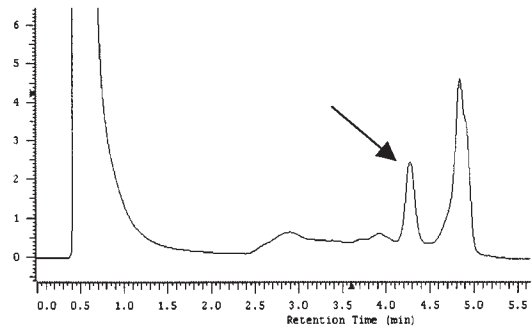
1. In the authors' laboratory, we find that the counterstain containing both DAPI and PI produces a banding pattern that allows ready identification of chromosome 17. To facilitate identification of chromosome 17, labeled DNA from a control probe may be added to the hybridization mixture. This could be from the short arm or distal long arm of chromosome 17.
2. For old slides, or slides that have a large amount of cytoplasm around the chromosomes, pretreatment in pepsin or collagenase may improve probe penetration. In addition, the slide denaturation time may be increased from 2–5 min.

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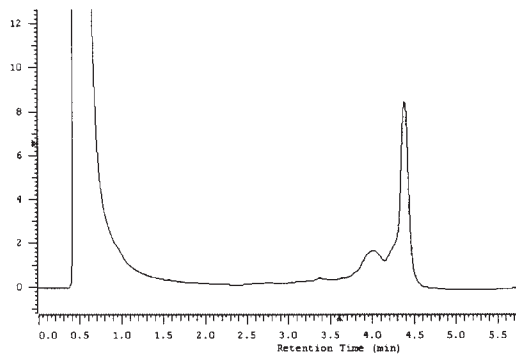
Fig. 2. (see facing page) DHPLC chromatograms for *NF1* mutations identified in exons 4b, 22, and 39.



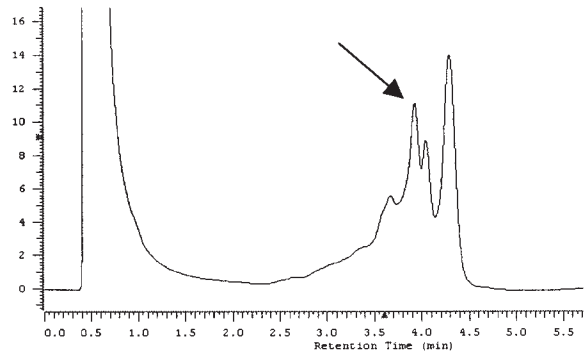
Exon4b, resolution temperature=54°C  
normal control



Exon4b, resolution temperature=54°C  
495delTGTT



Exon22, resolution temperature=58°C  
normal control



Exon22, resolution temperature=58°C  
R1241X

**Table 4****PCR Plate Setting for the Amplification of 60 Exons of *NF1* Gene Based on Six Test and Two Control (Positive and Negative) Samples**

Plate 1—set AT as 5.7°C + 9°C

	Injection	1	2	3	4	5	6	7	8	9	10	11	12
A	Water control												
B	Heterozygote												
C	Sample 1												
D	2	Exon 30	Exon 34	Exon 22	Exon 32	Exon 38	Exon 42	Exon 47	Exon 45	Exon 44	Exon 41	Exon 48a	Exon 48
E	3												
F	4												
G	5												
H	6												
(AT °C)		57.0	57.3	57.8	58.6	59.6	60.8	62.0	63.2	64.5	65.4	65.8	66.1

Plate 2—set AT as 53.5°C + 4°C

	Injection	1	2	3	4	5	6	7	8	9	10	11	12
A	Water control												
B	Heterozygote												
C	Sample 1												
D	2	Exon 25	Exon 27b	Exon 43	Exon 10b	Exon 4a	Exon 12a	Exon 9	Exon 19b	Exon 17	Exon 5	Exon 10c	Exon 23-1
E	3												
F	4												
G	5												
H	6												
(AT °C)		53.5	53.6	53.8	54.2	54.6	55.2	55.7	56.2	56.8	57.2	57.4	57.5

Plate 3—set AT as 57.5°C + 5°C

	Injection	1	2	3	4	5	6	7	8	9	10	11	12
A	Water control												
B	Heterozygote												
C	Sample 1												
D	2	Exon 29	Exon 2	Exon 24	Exon 26	Exon 27a	Exon 46	Exon 40	Exon 36	Exon 39	Exon 49	Exon 23a	Exon 8
E	3												
F	4												
G	5												
H	6												
(AT °C)		57.5	57.6	57.9	58.4	58.9	59.6	60.0	60.9	61.7	62.1	62.4	62.5



**Table 4 (continued)**

Plate 4—set AT as 58.0°C + 2°C

	Injection	1	2	3	4	5	6	7	8	9	10	11	12
A	Water control												
B	Heterozygote												
C	Sample 1												
D	2	Exon 37	Exon 4c	Exon 10a	Exon 12b	Exon 14	Exon 6	Exon 18	Exon 21	Exon 23-2	Exon 11	Exon 3	Exon 16
E	3												
F	4												
G	5												
H	6												
(AT °C)		58.0	58.0	58.1	58.3	58.5	58.8	59.1	59.3	59.6	59.8	59.9	60.0

Plate 5—set AT as 63.0 + 2°C

	Injection	1	2	3	4	5	6	7	8	9	10	11	12
A	Water control												
B	Heterozygote												
C	Sample 1												
D	2	Exon 9a	Exon 4b	Exon 20	Exon 13	Exon 7	Exon 28	Exon 31	Exon 33	Exon 35	Exon 19a	Exon 1	Exon 15
E	3												
F	4												
G	5												
H	6												
(AT °C)		63.0	63.0	63.0	63.3	63.5	63.8	64.1	64.3	64.6	64.8	64.9	65.0

3. A high background signal may be caused by a number of factors that include: the post-hybridization wash being of insufficient stringency, the probe fragments being too large (greater than 500 kb), and poor-quality metaphase chromosome spreads with surrounding cytoplasm. The first problem may be overcome by removing the cover slip, washing the counterstain off the slide with 4T buffer and replacing the slide in a higher-stringency solution such as 1X SSC or 0.5X SSC.
4. A weak or absent signal may be caused by a number of factors that include: insufficient denaturation of the probe or slide, a too stringent post-hybridization wash, insufficient probe concentration, and incorrect use of the detection reagents. A weak signal may be amplified by further application of biotinylated anti-avidin and fluorescein-avidin.
5. The Cot-1 DNA in the probe mixture acts as competitor DNA to suppress repetitive sequences that would interfere with the hybridization signal. The formamide reduces the melting temperature ( $T_m$ ) of the DNA and the dextran sulfate is an inert polymer which greatly increases the rate of hybridization.

6. Instead of incorporating indirectly labeled haptens into the DNA at nick translation, nucleotides directly labeled with a fluorochrome may be used.
7. If a deletion is found in a patient, parental bloods should be requested for analysis, as there are documented cases of asymptomatic mosaic carriers of the deletion passing on the deletion to their children. Analysis of 30 cells rules out a level of 10% mosaicism with 95% confidence.
8. All the buffer solutions should be made to analytical chemistry standards, using quality-assured glassware. For health and safety reasons, always wear gloves when making buffers. Whenever possible, use water of the highest quality—e.g., Millipore water. The minimum standard is HPLC-grade water. The smaller the amount of transition ions in the water, the more prolonged the column's life will be.

Before use, the Duran bottles should be washed out with Millipore water to remove any transition ions, especially if the glassware has been autoclaved.

9. Accurate and consistent compositions are critical when preparing buffers A and B. Elution times are dependent on accurately prepared buffers. Incorrectly prepared buffers could also cause samples to elute early or late.
10. Evaporation will lead to an increase in ionic strength and volatility of the ACN. Both conditions will affect the performance of the instrument.
11. Buffers A and B must be freshly prepared within 1 wk. Buffer C must be changed every 3 wk.
12. The column should be well-equilibrated before the initiation of analysis the first injection. It may be necessary to set up at least one blank before the first injection.
13. A column can be maintained on the instrument for 2–4 d by pumping the column at a sleep condition (flow rate 0.05 mL/min with 50% buffer A and 50% buffer B). Prior to long-term storage, pump the column with 50% ACN for 30 min at 0.75 mL/min to prevent bacterial growth. Disconnect the column and tightly seal it with end fittings to prevent drying of the bed.
14. If the column is new or has been stored off the instrument, flush the column with 50% buffer A, 50% buffer B, at 0.5 mL/min<sup>-1</sup> for at least 45 min, then gradually increase the flow rate from 0.5, .75, and finally to 0.9 mL/min<sup>-1</sup>. Run three blank gradients before the first analytical run. Recalibration may be necessary by using DNA mol-wt markers (size standard), as well as a mutation standard.
15. Do not start the buffer flow until the oven and the column have reached at least 40°C. Once the temperature is reached, the flow may be started at 0.5 mL/min. An additional 10–15 min will be required for the temperature and pressure to stabilize.
16. After fitting a new peak inline filter. Disconnect the column and flush the peak inline filter with 100% buffer B at 0.5 mL/min for at least 10 min before re-fitting the column.
17. Wash the injection valve and needle before starting a series of runs. Press the “wash” key on the key pad on the front of the auto sampler. Washing solution will be taken up by the syringe in the auto sampler. Then push through the injection valve and out through the wasted line. Repeat this step until you see liquid empty through the waste line into the waste bottle.

18. For every 100 injections, to increase the life of the column, it is advisable to wash the column with 75% ACN at 65°C. Before starting a new analysis, an equilibration for at least 10 min is required to balance the column back to a normal condition.

## Acknowledgments

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## Duchenne and Becker Muscular Dystrophy

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### 1. Introduction

The two forms of dystrophin-associated muscular dystrophies, known as Duchenne and Becker muscular dystrophy (DMD/BMD; OMIM 310200) are caused by genetic defects in the huge DMD gene (79 exons), located at Xp21 and coding for the 427-kDa protein known as dystrophin (1,2). DMD is the most frequent muscular disorder in boys, with an incidence of 1 in 3,500 males and a new mutation frequency of 1 in 10,000. Characteristics of the severe DMD phenotype are early-onset progressive degenerative myopathy with pseudohypertrophy of the calves, associated with highly elevated creatine kinase levels in blood and the almost complete absence (<2%) of dystrophin in muscle cells. Although dystrophin is detectable in patients with the milder Becker phenotype, the protein is less functional and is usually of abnormal size (3).

The majority of mutations, found in the DMD gene in approx 65% of patients, are deletions or duplications of one or more exons, with hot spots in the 5' part (exons 3–20) and the central part (exons 44–55) of the gene (4). Since diagnostic tools became available to perform complete gene scans, it has been shown that the genetic defect in the remaining set of patients are point mutations and small deletions or insertions. These small mutations are identified throughout the gene in almost all exons with only few mutations reported more than once (5,6).

#### 1.1. DNA Diagnosis in BMD/DMD Patients (see also Fig. 1)

Patient samples are sent in for confirmation of the clinical diagnosis or as key samples to be able to perform carrier detection for female relatives. The first method to start with is a multiplex PCR deletion analysis, since 60% of the patients have a deletion of one or more exons. To identify a deletion in the

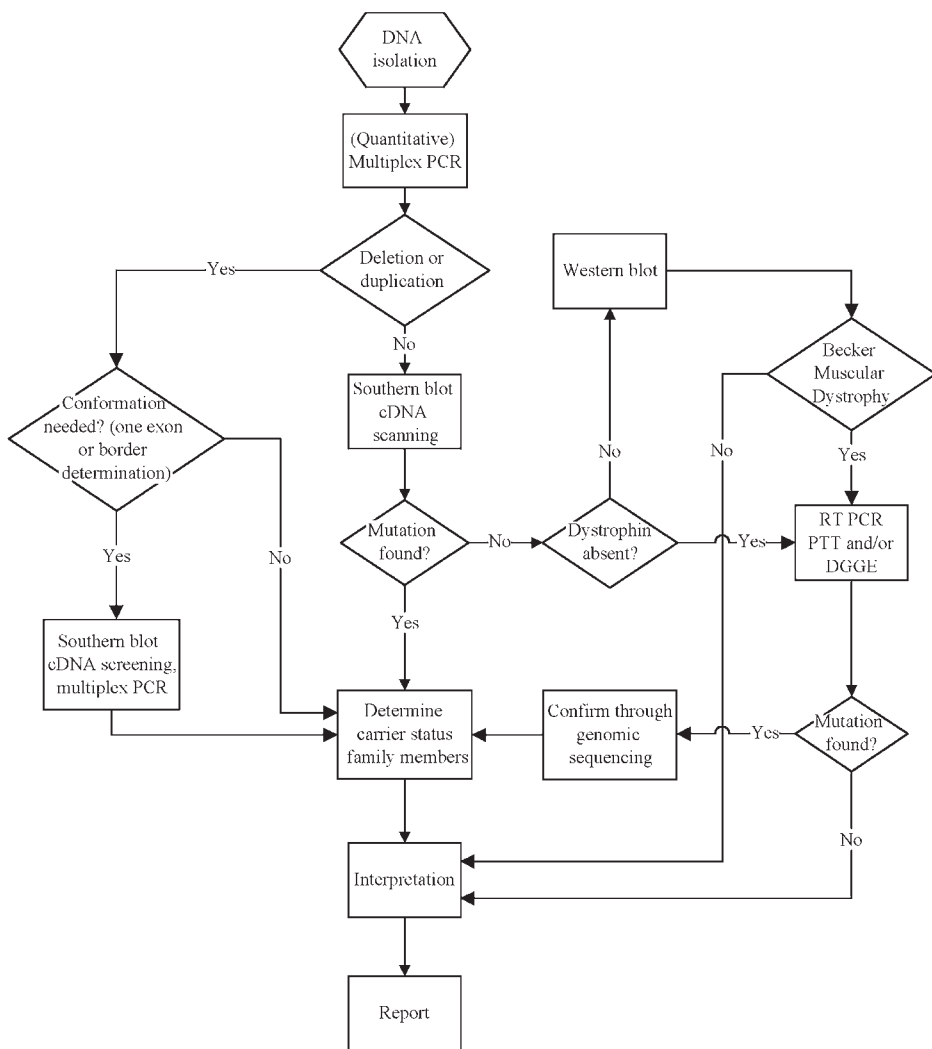


Fig. 1. Strategy for DNA analysis in BMD/DMD patients

patient's DNA, two multiplex PCRs were constructed in which 9 exons can be amplified simultaneously, and 98% of all deletions could be identified (*see Tables 1 and 2*) (7,8). After PCR and agarose gel electrophoresis, the absence of amplicons in the gel can simply be scored as a deletion of the corresponding exon (*see Fig. 2*).

To identify a duplication, detected in approx 5% of the patients, or to further characterize a deletion, the quantitative multiplex PCR can be used (9).



**Table 1**  
**Primer Sequences for the Multiplex PCR Deletion Screening, Kit 1 (8)**

Exon	F/R	Sequence 5'–3'	Size
45	F	AAACATGGAACATCCTTGTGGGGAC	547
	R	CATTCTATTAGATCTGTCGCCCTAC	
48	F	TTGAATACATTGGTTAAATCCCAACATG	506
	R	CCTGAATAAAGTCTTCCTTACCACAC	
19	F	TTCTACCACATCCCATTTTCTTCCA	459
	R	GATGGCAAAAGTGTTGAGAAAAAGTC	
17	F	GACTTTCGATGTTGAGATTACTTTCCC	416
	R	AAGCTTGAGATGCTCTCACCTTTTCC	
51	F	GAAATTGGCTCTTTAGCTTGTGTTTC	388
	R	GGAGAGTAAAGTGATTGGTGGAAAATC	
8	F	GTCCTTTACACACTTTACCTGTTGAG	360
	R	GGCCTCATTCTCATGTTCTAATTAG	
12	F	GATAGTGGGCTTTACTTACATCCTTC	331
	R	GAAAGCACGCAACATAAGATACACCT	
44	F	CTTGATCCATATGCTTTTACCTGCA	268
	R	TCCATCACCTTCAGAACCCTGATCT	
4	F	TTGTCCGTCTCCTGCTGGTCAGTG	196
	R	CAAAGCCCTCACTCAAACATGAAGC	

With two sets of primers, one for the 5' part of the gene containing 13 amplicons and one for the 3' part containing 14 amplicons, a quantitative PCR analysis of 18 cycles of PCR is performed (see **Fig. 3** and **Tables 3** and **4**). The forward primers in the multiplex contain fluorescent labels to allow the use of an automated sequencer for detection and quantitation of the PCR products. After electrophoresis, the peak areas of all amplicons of samples of interest are compared to those of normal controls by calculating the Dosage Quotients (DQ) values:

$$DQ^{Pm/2} = \frac{(\text{control Pm peak area} / \text{control exon 2 peak area})}{(\text{sample Pm peak area} / \text{sample exon 2 peak area})}$$

The theoretical DQ values are given in **Table 5**, compared to those found in practice using a panel of 100 female carriers. Mutations can be scored by determining whether the DQ values of one exon, compared to all other exons fall in the normal, deletion, or duplication range (see **Fig. 4**).

To determine the extent of a deletion or duplication (if not conclusive after multiplex PCR) or to complete the search for deletions and duplications in the gene, a quantitative Southern blot analysis using cDNA probes (**10**) can be per-

**Table 2**  
**Primer Sequences for the Multiplex PCR Deletion Screening, Kit 2 (7)**

Exon	F/R	Sequence 5'–3'	Size
Pm	F	GAAGATCTAGACAGTGGATACATAACAAATGCATG	535
	R	TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC	
3	F	TCATCCATCATCTTCGGCAGATTAA	410
	R	CAGGCGGTAGAGTATGCCAAATGAAAATCA	
43	F	GAACATGTCAAAGTCACTGGACTTCATGGA	357
	R	ATATATGTGTTACCTACCCTTGTCGGTC	
50	F	CACCAAATGGATTAAGATGTTCATGAAT	271
	R	TCTCTCTCACCCAGTCATCACTTCATAG	
13	F	AATAGGAGTACCTGAGATGTAGCAGAAAT	238
	R	CTGACCTTAAGTTGTTCTTCCAAAGCAG	
6	F	CCACATGTAGGTCAAAAATGTAATGAA	202
	R	GTCTCAGTAATCTTCTTACCTATGACTATGG	
47	F	CGTTGTTGCATTTGTCTGTTTCAGTTAC	181
	R	GTCTAACCTTTATCCACTGGAGATTTG	
60	F	AGGAGAAATTGCGCCTCTGAAAGAGAACG	139
	R	CTGCAGAAGCTTCCATCTGGTGTTTCAGG	
52	F	AATGCAGGATTTGGAACAGAGGCGTCC	113
	R	TTCGATCCGTAATGATTGTTCTAGCCTC	

formed. Southern blot analysis is also advised to confirm a single exon deletion or duplication found by multiplex PCR to rule out rare polymorphisms in the primer sites. As an alternative to Southern blotting, a different multiplex PCR with other primers can be used as well. Currently the Multiplex Amplifiable Probe Hybridization (MAPH) technique (*11*) is being set up for the DMD gene to study all 79 exons simultaneously for deletions and duplications.

To identify a possible small mutation, in the absence of any gross rearrangements, many of the mutation scanning techniques that have been successful in DNA diagnostics are also used to study the DMD gene. Before the relatively costly and laborious mutation scanning is initiated, it is advisable to use immunohistochemistry and/or a Western blot for dystrophin study on muscle tissue to confirm the clinical diagnosis (*12*). Although methods such as heteroduplex analysis (HA) (*13*) and single-strand conformation analysis (SSCA) (*14*) have identified mutations, the limited sensitivity of the methods and the need to use several electrophoretic conditions for SSCA to increase sensitivity is often seen as a drawback. Denaturing gradient gel electrophoresis (DGGE) is much more sensitive, and can—when set up in a multiplex fashion using uni-

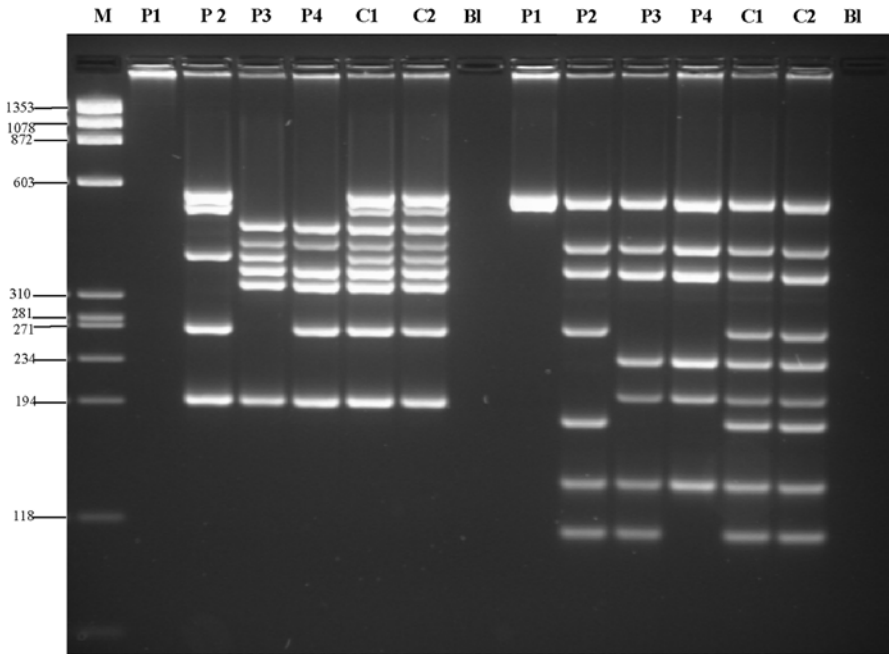


Fig. 2. Gel Image of the two multiplex PCR assays for deletion detection in patients. For Patient 1, a deletion of all DMD exons with the exception of exon 1 is detected (amplicon Pm is present). Patient 2 shows a minimal deletion of exons 8 to 19. In Patient 3, the exact deletion of exons 44 to 50 is characterized using this multiplex because exons 43 and 51 are present. Patient 4 has a minimal deletion of exons 45 to 52, the deletion may extend further at the 3' end (exons 53 and beyond). Note that for all four patients, the observed deletion confirms the results of both multiplex PCRs.

form conditions and robotics—provide a high-throughput mutation scanning assay. A multiplex DGGE was developed for the DMD gene in which 95 amplicons (see **Table 6**) are analyzed in multiplex using one PCR program and only four different DGGE conditions (**6**). Reverse transcriptase (RT)-PCR followed by protein truncation test (PTT) analysis on RNA from muscle biopsies of patients have been shown to be successful because many frameshift, non-sense, and splice-site mutations have been identified using this approach (**15**). However, it can be difficult to set up and use these RNA-based techniques on a routine basis for diagnostic purposes.

When the full extent of a deletion or duplication is determined, or the causative small mutation is detected, it is often possible to predict an expected phenotype from the observed genotype, since in-frame deletions or duplications (the reading frame remains intact) is usually associated with a Becker phenotype,

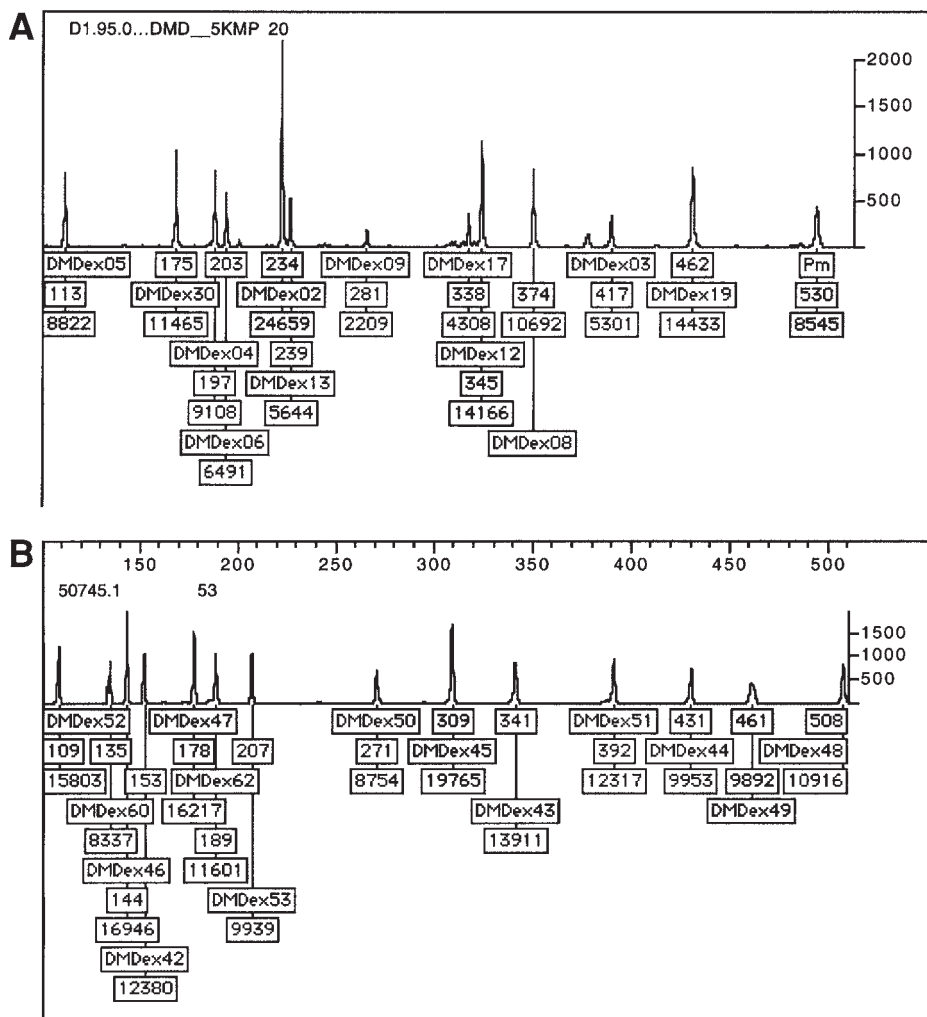


Fig. 3. Electropherograms of 5' (A) and 3' (B) multiplex kits, analyzed in the GenoTyper software.

whereas out-frame mutations (leading to a premature stop) are found in patients with a Duchenne phenotype (16).

## 1.2. Carrier Detection

After identification of the causative mutation in the patient, carrier detection is directly feasible using the quantitative multiplex PCR analysis in case of a deletion or duplication, or a direct mutation detection technique such as direct

**Table 3**  
**Primer Sequences for the 5' Quantitative Multiplex PCR Screening Kit**

Exon	F/R	Sequence 5'–3'	Size	Size calling in GeneScan (avg)
Pm	F	TAGACAGTGGATACATAACAAATGCATG	535	529
	R	TTCTCCGAAGGTAATTGCCTCCCAGATCTGAGTCC		
19	F	TTCTACCACATCCCATTTTCTTCCA	459	461
	R	GATGGCAAAAGTGTGAGAAAAAGTC		
3	F	TCATCCATCATCTTCGGCAGATTAA	410	416
	R	CAGGCGGTAGAGTATGCCAAATGAAAATCA		
8	F	GTCCTTTACACACTTACCTGTTGAG	360	371
	R	GGCCTCATTCTCATGTTCTAATTAG		
12	F	GATAGTGGGCTTACTTACATCCTTC	331	342
	R	GAAAGCACGCAACATAAGATACACCT		
17	F	GCTATTTTGATCTGAAGGTCAATCTACC	327	335
	R	AAGCTTGAGATGCTCTCACCTTTTCC		
9	F	TCTATCCACTCCCGAACCTCTCTGCAG	278	280
	R	AACAAACCAGCTTTCACGAGGAGA		
13	F	AATAGGAGTACCTGAGATGTAGCAGAAAT	238	238
	R	CTGACCTTAAGTTGTTCTTCCAAAGCAG		
2	F	CACTAACACATCATAATGGAA	233	233
	R	GATACACAGGTACATAGTCCA		
6	F	CCACATGTAGGTCAAAAATGTAATGAA	202	203
	R	GTCTCAGTAATCTTCTTA CCTATGACTATGG		
4	F	TTGTCCGGTCTCCTGCTGGTCAGTG	196	197
	R	CAAAGCCCTCACTCAAACATGAAGC		
30	F	AGGCTGTAAGGAGGCAAAAGTTGC	175	175
	R	GATGTACTTGCCTGGGCTTCCCTGAGGC		
5	F	GGTTGATTTAGTGAATATTGGAAGTAC	114	113
	R	CCATTCATCAGGATTCTTACCTGCC		

sequencing when a small mutation has been identified. Techniques such as DGGE or SSCA are not advisable for direct carrier detection because these are indirect techniques, and results might be hampered by polymorphisms. When patient material is not available for mutation scanning, DNA of an obligate carrier, or female at-risk can be used for the quantitative multiplex PCR or the multiplex DGGE analysis.

Haplotype analyses using STR markers to determine the at-risk haplotype in the family is the only alternative when a mutation is not identified in the patient or carrier (at risk) (17,18). To perform the analyses, it is imperative that

**Table 4**  
**Primer Sequences for the 3' Quantitative Multiplex PCR Screening Kit**

Exon	F/R	Sequence 5'-3'	Size	Size calling in GeneScan (avg)
48	F	TTGAATACATTGGTTAAATCCCAACATG	506	507
	R	CCTGAATAAAGTCTTCCTTACCACAC		
49	F	GTGCCCTTATGTACCAGGCAGAAATTG	439	445
	R	GCAATGACTCGTTAATAGCCTTAAGATC		
44	F	GTTGTGTGTACATCGTAGGTGTGTA	426	433
	R	TCCATCACCCCTCAGAACCTGATCT		
51	F	GAAATTGGCTCTTTAGCTTGTGTTTC	388	397
	R	GGAGAGTAAAGTGATTGGTGAAAATC		
43	F	GAACATGTCAAAGTCACTGGACTTCATGG	357	342
	R	ATATATGTGTACCTACCCTTGTCGGTCC		
45	F	CTTTCTTTGCCAGTACAACCTGCATGTG	307	316
	R	CATTCCCTATTAGATCTGTCGCCCTAC		
50	F	CACCAAATGGATTAAGATGTTTCATGAAT	271	272
	R	TCTCTCTCACCCAGTCATCACTTCATAG		
53	F	TTGAAAGAATTCAGAATCAGTGGGATG	212	211
	R	CTTGGTTTCTGTGATTTTCTTTTGGATTG		
62	F	GTCTTTCCTGTTTGCGATGAATTTGACC	191	193
	R	CTCACTTGTGAATATACAGGTTAGTCAC		
47	F	CGTTGTTGCATTTGTCTGTTTCAGTTAC	181	182
	R	GTCTAACCTTTATCCACTGGAGATTTG		
42	F	CACACTGTCCGTGAAGAAACGATGATG	155	156
	R	TTAGCACAGAGGTCAGGAGCATTGAG		
46	F	GCTAGAAGAACAAGAATATCTTGTC	148	147
	R	CTTGACTTGCTCAAGCTTTTCTTTTAG		
60	F	AGGAGAAATTGCGCCTCTGAAAGAGAACG	139	138
	R	CTGCAGAAGCTTCCATCTGGTGTTTCAGG		
52	F	AATGCAGGATTTGGAACAGAGGCGTCC	113	110
	R	TTCGATCCGTAATGATTGTCTAGCCTC		

the DNA of key family members is available. A total of 19 intragenic and 8 flanking STR markers are currently available for haplotype analysis (*see Table 7*). Because of the high recombination frequency of approx 10% across the gene (*19*) at least three markers, one intragenic and one at either side of the gene are needed to reduce the risk of missing a double recombinant. In our laboratory, a set of eight highly informative markers is used for the initial analy-

**Table 5**  
**DQ Values, Theoretical and as Determined in Practice**

	DQ value	
	Theoretical	Practice
Normal	1.0	0.67–1.50
Duplication in patient	0.5	nd
Duplication in patient	2	nd
Deletion in carrier	0.5	0.33–0.70
Deletion in carrier	2	1.42–2.76
Duplication in carrier	0.67	0.51–0.77
Duplication in carrier	1.50	1.45–1.97

nd, not determined

ses, which usually provides enough information for the haplotype analyses and risk calculations for female relatives. In some cases, an intragenic recombination is observed, which highlights the major drawback of this technique.

Another complicating factor is the presence of a possible germline mosaic in apparent *de novo* cases, which must always be accounted for in the risk calculations for carriers (20). The absence of the causative mutation in somatic DNA of the mother of a patient therefore only reduces the risk for a second affected son to 7%. Also, grand-paternal mosaicism should be taken into consideration for risk calculations of maternal aunts of the patients.

### 1.3. Prenatal Diagnosis

After determination of a certain carrier risk, many couples choose a prenatal diagnosis if a male fetus is concerned. Once the mutation has been identified in the family, the analysis is relatively straightforward using either the (quantitative) multiplex PCR or direct sequencing to determine the presence or absence of the mutation. However, when the mutation is not found in the DNA sample of the fetus, a possible maternal contamination of the chorion villi should always be excluded by the use of informative markers.

In the case of a male fetus with an at-risk haplotype, the MyoD-induced myo-differentiation technique can be used (21). By introducing the MyoD-gene in non-muscle cells such as chorionic villus, cells are forced into myogenic differentiation. Immunohistochemical analysis of these converted cells will reveal the presence or absence of dystrophin. In combination with RT-PCR and PTT mutation analysis, this technique will provide a definite answer regarding the genetic status of the fetus at risk.

**A**

Sample: D1.01.01504

Control: D2.00.00881 D2.00.02318

Remarks: female, duplication exons 12 and 13

EXONS	SAMPLE	CONTROL	2	3	4	5	6	8	9	12	13	17	19	30
Pm	16,462	14,783	0.95	0.99	0.96	0.97	0.92	1.00	0.91	1.53	1.54	1.01	0.94	0.88
2	42,538	40,218		1.04	1.01	1.02	0.97	1.05	0.96	1.61	1.62	1.06	0.99	0.93
3	9,710	8,792			0.96	0.97	0.93	1.01	0.92	1.54	1.55	1.01	0.95	0.89
4	14,087	13,223				1.01	0.96	1.04	0.96	1.60	1.61	1.05	0.99	0.92
5	11,364	10,569					0.95	1.03	0.95	1.58	1.59	1.04	0.98	0.91
6	10061	9,835						1.09	1.00	1.66	1.68	1.10	1.03	0.96
8	20,008	17,994							0.92	1.53	1.54	1.01	0.94	0.88
9	3447	3,387								1.67	1.68	1.10	1.03	0.96
12	39456	23,182									1.01	0.66	0.62	0.58
13	15,254	8,895										0.65	0.61	0.57
17	7,886	7,040											0.94	0.88
19	25,317	24,123												0.93
30	11,227	11,449												

**B**

Sample: D2.00.02621

Control: D2.00.00881 D2.00.02318

Remarks: female, deletion exon 45-52

EXONS	SAMPLE	CONTROL	43	44	45	46	47	48	49	50	51	52	53	60	62
42	19,210	12,147	0.97	0.96	0.47	0.48	0.47	0.45	0.49	0.56	0.47	0.56	0.96	0.87	0.98
43	39,088	25,481		0.99	0.49	0.49	0.49	0.46	0.50	0.58	0.49	0.58	1.01	0.90	1.01
44	12,634	8,354			0.49	0.50	0.50	0.47	0.51	0.58	0.50	0.58	1.03	0.91	1.02
45	17,724	23,775				1.01	1.01	0.95	1.04	1.19	1.01	1.18	2.08	1.84	2.08
46	11,228	14,938					1.00	0.94	1.03	1.18	1.00	1.17	2.06	1.83	2.06
47	11,811	15,746						0.95	1.03	1.18	1.00	1.18	2.07	1.83	2.07
48	10,398	14,656							1.09	1.25	1.06	1.24	2.19	1.94	2.18
49	10,909	14,086								1.14	0.97	1.14	2.00	1.78	2.00
50	4,564	5,166									0.85	1.00	1.75	1.56	1.75
51	14,913	19,879										1.18	2.07	1.83	2.07
52	8,022	9,084											1.76	1.56	1.76
53	15,219	9,817												0.89	1.00
60	11,410	8,299													1.13
62	17,229	11,115													

Fig. 4. Calculation of Dosage Quotients (DQ values) for the 5' and 3' quantitative multiplex kit. (A) Example of the DQ values of a duplication for exons 12 and 13 detected in a carrier using the 5' multiplex kit. (B) Example of the DQ values of a deletion for exons 45 to 52 detected in a carrier using the 3' multiplex kit.



**Table 6**  
**Primers for DGGE Analysis of the Dystrophin Gene**

Exon	Sequence forward primer 5'-3'	Sequence reverse primer 5'-3'PCR	Length
1m	AGCCTACTGGAGCAATAAAG	[clamp]-AGCTTGTACAAACTAAACG	209
2	[clamp]-ATGGAAAGTACTTTGGTTG	CAGGTACATAGTCCATTTTG	252
3A	[clamp]-TAAATGAGTGTATTTTTTT	TTTTGCCCTGTCAGGCCTTC	158
3B	CTTCAGTGACCTACAGGATG	[clamp]-TTAACTTTCTTAAAAATAAG	135
4	[clamp]-CTCTCTGCTGGTCAGTGAAC	GTGTGTCACAGCATCCAGAC	200
5	[clamp]-GCATTTGGTCTCTTACCTTC	TTTGTTCACACGTCAAGGG	259
6A	[clamp]-GGTTCTTGCTCAAGGAATGC	GCTCAGGAGAATCTTTTCAC	161
6B	AAATATCATGGCTGGATTGC	[clamp]-ATCAGAGTCTAAATCACCAC	253
7	GCGCGTTTGTCTTTGTGTATGTGTG	[clamp]-TACTAAAAGCAGTGGTAGTC	232
8	GCGCGCCAAAAATTGATGTGTAGTG	[clamp]-ATCTTGAATAGTAGCTGTCC	291
9	CCCTCCTCTATCCACTCC	[clamp]-GGAAGCAGTTCTCTGGTTTG	255
10	GCGCGGACATTAATTGTGTAACACC	[clamp]-GGATGACTTGCCATTATAAC	330
11	CACCGATTACCTAGAGTTC	[clamp]-TCCAAAACTTGTTAGTCTTC	320
12	[clamp]-AAGTTGCTTTCAAAGAGGTC	GTACTATACACAGAGTTTGC	294
13A	[clamp]-CATTCAACACACATGTAAG	AGCAGCAGTTGCGTGATCTC	202
13B	CTCTCACTCACATGGTGGTG	[clamp]-CTTTTAAAGGACATATTTAG	160
14	GCGCGGTTTGTGCTGATGCTTGATTG	[clamp]-CCCCGTGTCTTTTACAGCTA	220
15	TATGTTTATTTATTCCTTGG	[clamp]-TACTGGGTTTTTATAAGACC	231
16	[clamp]-TGCAACCCAGGCTTATTCTG	GCGCGTCTCTGAGATAGTCTGTAGC	302
17	AGCAATGTCTGACCTCTGTT	[clamp]-GCTGTAAATGAGTTTCTCC	308
18A	[clamp]-AGAAGAAAGAGATAATCAAG	AGTTGCCTTCCTTCCGAAAAG	189
18B	CTGGATTACTCGCTCAGAAG	[clamp]-GCAGCACAAAATGAGTACAG	179
19	GCGCGGATTCACAGTCCCTTGATTG	[clamp]-GTGTTTATCAAATCCCTAAG	227
20A	[clamp]-TCAGTCTGTGGGTTCAAGGG	CAGTAGTTGTCATCTGCTCC	253
20B	GGCTGGAGTATCAGAACAAC	[clamp]-GGAATGCCAAGAAATACCTA	248
21	ATGTATGCAAAGTAAACGTG	[clamp]-TGGAAAATGTCAAGTTAGCC	310
22	[clamp]-CAATTAAGTGATTCTCATT	GCGTGCTTTATTGTTTTGAC	264
23A	[clamp]-TTCATCAATTAGGGTAAATG	AGGAGAGCTTCTTCCAGCGT	233
23B	CGCCCTCTGAAATTAGCCGG	[clamp]-GTAATAAAAAATGAGGGTAG	214
24	AATCAGCACACCAGTAATGC	[clamp]-TTATTCATATTAAGGCATC	231
25	[clamp]-TTTTACGTGGCTTAAATTG	TTAAGTACGTTGAGGCAAGC	274
26A	[clamp]-CATCACTGTCAATAATCGTG	TCAAGATACTTCTTTCAGC	197
26B	CTATCAGAGATGCACGAATG	[clamp]-TTTACCTTCATCTTCAAC	153
27	[clamp]-TTTCATGCTATTAAGAGAGC	AACTATGACCATGTATTGAC	310
28	[clamp]-TTCACATTTACTTTTCTACC	CTGCATATAAATCATCATCA	273
29A	[clamp]-CATTTGCTGATAATCCAATG	ATTAGCTCATCCATGACTCC	206
29B	ATTCGCATATTGGCACAGAC	[clamp]-ATGCAAATTAGATTAAGAG	186
30	[clamp]-TACAGAAAAGCTATCAAGAG	CAAACAAAAAGAATGGAAGC	301

(continued)

Table 6 (continued)

Exon	Sequence forward primer 5'-3'	Sequence reverse primer 5'-3'PCR	Length
31	[clamp]-GTTAGTTGTTCTTTGTAGAG	GCCCAACGAAAACACGTTCC	246
32	[clamp]-GGCAAATTAATCAGTGCC	TAATGAGGAAAGTCAAGGGG	305
33	[clamp]-TAACTACTGATTATCATG	GTGGAAAGAAGTGTGTTGTGG	276
34	[clamp]-ATTTGAATTAAGAGTAAAC	ATGTGTTTTACGATATGTTT	310
35	[clamp]-CAAGACATTACTTGAAGGTC	CGCGCACAGAGAAGGGTGTAAAAGC	298
36	GTCTAACCAATAATGCCATG	[clamp]-GTAAGTGGTGTACAATTTGG	258
37	[clamp]-CTAACTTCAAGTCTATCTC	CGCAAGAGACCATTTAGCAC	298
38	[clamp]-CTTTGGTTTATGTTTCTTA	TTTATTTCCACTCCTAGTTC	275
39	CTCTGTAAACAATGTACAGC	[clamp]-AAACCACAGGCAAGGTATAT	283
40	GCGCGATTTTAATAATGTCTGCACC	[clamp]-AAAATCTGGTATTGACATTC	255
41A	[clamp]-ATGTGGTTAGCTAACTGCC	GCTGGATCTGAGTTGGCTCC	203
41B	GAAGAAAGAGGAGCTGAATG	[clamp]-AGTTGCAAAACACATACGTGG	240
42	[clamp]-TAATGGAGGAGGTTTCACTG	GCGCCACCTTGTAATAACGAATG	336
43A	[clamp]-CTATAGACAGCTAATTCATT	GCTGCTGTCTTCTTGCTATG	159
43B	AGTCTACAACAAAGCTCAGG	[clamp]-TCATTTCTGCAAGTATCAAG	242
44	[clamp]-CGCTATATCTCTATAATCTG	TAAAGAGTCCAGATGTGCTG	317
45	[clamp]-GACATGGGGCTTCATTTTTG	GTTTTCAITTCCTATTAGATC	291
46	[clamp]-GTTTGAGAACTATGTTGGAA	ACTTCTTTATGCAAGCAGGC	289
47	AGGTAGTTGGAATTGTGCTG	[clamp]-GCACCCAGGAAAACAAAATAC	324
48	[clamp]-GGCTTATGCCTTGAGAATTA	CAGTGATATTGCCATTTTTTC	336
49	[clamp]-ACTGTGAAGTTAATCTGCAC	CAACAGGCGAAGCATAACCC	243
50	[clamp]-CGAATAAGTAATGTGTATGC	TTGAACAAATAGCTAGAGCC	229
51A	[clamp]-TCTTCTTTTTTCTTTTTGTC	GATCAAGCAGAGAAAAGCCAG	243
51B	GATTTCAACCGGGCTTGAC	[clamp]-AAAGAAAAACTTCTGCCAAC	204
52	GAAGAACCCTGATACTAAGG	[clamp]-CATCTTGCTTTGTGTGTC	257
53A	[clamp]-TCCTCCAGACTAGCATTTAC	AGACCTGCTCAGCTTCTTCC	217
53B	TTCAGAACCGGAGGCAACAG	[clamp]-CAAATGTAACCAGTATTTTA	257
54A	[clamp]-AAAAACTGACATTCATTCTC	TAATCCCGGAGAAGTTTCAG	160
54B	CCAGTGGCAGACAAATGTAG	[clamp]-CCCATTATTACAGCCAACAG	222
55	[clamp]-TTTATGGAGTTCACTAGGTGC	ACAAATGCTGAGAATTGTTT	362
56	TCTGCACATATTCTTCTTCC	[clamp]-ATTTGTGGCCTTTTTGCTCC	280
57	GATATTCTGACATGGATCGC	[clamp]-GTCAGTGGATTACTATGTGC	298
58	TTGAGAAGAATGCCACAAGC	[clamp]-TCACCACTGATCCTTCTATC	267
59	[clamp]-AAAGAATGTGGCCTAAAACC	GCGGCCGCCAAAAGGCCCTGAAG CAAAG	382
60	ATTCTCATCTTCCAATTTGC	[clamp]-TTACTGTAACAAAGGACAAC	266
61A	[clamp]-GAATGAGAGAACATAATTTT	TGAGATGCTGGACCAAAGTC	142
61B	GTCGAGGACCGAGTCAGGCA	[clamp]-AGATGCAATAAAGTTAAGTG	155

(continued)

Table 6 (continued)

Exon	Sequence forward primer 5'-3'	Sequence reverse primer 5'-3'PCR	Length
62	ATTTGACCTCCTTGCCTTTC	[clamp]-GTTAGTCACAATAAATGCTC	192
63	[clamp]-GTTTTCTTGACTACTCATGG	CCTGTCATTTAACTTGGAGG	199
64	GGCAAATCACTGGGCGTCGG	[clamp]-AGCAAAGACATAGTATCAAG	242
65	[clamp]-GGAAGTTTTACTCTTTGAG	CTAAGCCTCCTGTGACAGAG	340
66	GGTCTAGTAATTGTTTTCTG	[clamp]-CTAGGGTAATTAGCCAAACAT	221
67	TTGGATGTCAGGTTCTGCTG	[clamp]-GAAAGAATAAATATGTTACC	309
68A	[clamp]-CTTTCCTTTCATCCTTTGGC	TTGGACACTCTTTGCAGATG	244
68B	AGAAACTGCCAAGCATCAGG	[clamp]-AGGAGATAAAAAGATCAAGTC	181
69	[clamp]-CGAAGAAATACATACGTGTT	TGAACTAACTCTCACGTCAG	228
70	[clamp]-AGTTTTGAAATCATCCTGTC	CATCAAACAAGAGTGTGTTCTG	269
71	AGAAAGCGTGTGTCTCCTTC	[clamp]-AGCGAATGTGTTGGTGGTAG	172
72	GATGGTATCTGTGACTAATC	[clamp]-TCAATCAATATTTGCCTGGC	181
73	[clamp]-TCACATAAGTTTTAATGAGC	ACCTCTAAATCCCCTCAAAGC	210
74	[clamp]-TGAGTCCCTAACCCCAAAG	GCACTCTGCATACCAATGAC	307
75A	[clamp]-TTTACTTTTTTGATGCC	CCTTTGTGTTGACGCAGTAG	218
75B	CGCCGCCGGCCAATAGGAATCTGC AAGC	[clamp]-GTTTGTA AAAATCCCATCTC	321
76	[clamp]-TGAAAAGTAATTCTGTTTTTC	TCTACCTTTCTTCAGACAAC	247
77	[clamp]-ATGTAATTTTCCATTATTTG	GATCCCAGCAAATCTGAGTC	212
78	[clamp]-CCCTTCTGATATCTCTGCC	AAGCAGGATGAGACAGACAG	167
79	[clamp]-TGATGCTATCTATCTGCACC	TTCTGCTCCTTCTTCATCTG	193

[clamp] = is GC-clamp tail sequence added to the 5'-end of the primer (5'-CGCCCGC CGCGCCCGCGCCCGGCCGCGCCGCGGCCGC-3')

## 2. Materials

### 2.1. Multiplex PCR

1. Taq polymerase (Promega).
2. Bovine serum albumin (BSA), DNase-free, Amersham Pharmacia Biotech.
3. dNTP set, 100 mM deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate (dATP, dCTP, dGTP, dTTP), Amersham Pharmacia Biotech.
4. Multipurpose agarose (Roche Diagnostic).
5. Nusieve Agarose FMC (SanverTech).
6. 1 M Tris-HCl (pH 7.5) (Life Technologies).
7. 5X PCR-buffer: 83 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 335 mM Tris-HCl pH 8.8, 33.5 mM MgCl<sub>2</sub>, 34 μM ethylenediaminetetraacetic acid (EDTA) and 50 mM β-mercapto-ethanol. Store at -20°C for a maximum of 2 yr.

**Table 7**  
**Intragenic and Flanking STR Markers for Haplotyping of the DMD Gene**

Marker id	Literature id	Location	Heterozy- gosity%	allele range (bp)	PCR conditions		Primer sequences 5'-3'
					Alleles CEPH 134702	Annealing temp (°C) PCR buffer	
DXS 1068	AFM238yc11	Xp 11.4	67	245-259	251-253	60 PT	F CCTCTAAAGCATAGGGTCCA R CCCATCTGAGAACACGCTG
5' DYS 8	DXS 8025	Xp11.4	76	181-201	185-199	60 PT	F AGCTGGGCAGCAGAGC R TGGGTAATTCTTTGACATCACTC
DXS 8090	AFM338xa5	Xp 21	77	154-172	164-168	65 PT	F GGGTGAAATTCATCACAAA R ACAAATGCAGATGTACAAAAAATA
5' DYS 1*	DMD.PCR10	3,5 kb 5' e1 b.	60	175-185	175-181	60 PT	F ACTGTAAATGAAATTGTTTTCTAAGTGCC R GTTAACAAAATGTCCTTCAGTTCTATCC
5' DYS 2*	DMD.PCR11	1,2 kb 5' e1 b.	77	207-228	218-222	55 PT	F TCTTGATATATAGGGATTATTTGTGTTTGTATAC R ATTATGAAACTATAAGGAATAACTCATTTAGC
5' DYS 3	DMD.PCR12	3,5 kb 3' e1 b.	58	219-225	213-216	60 PT	F TTTTTAGGTATAACTTACATACAATAAACC R GTGACAATAAGCATATCAGTGGCTGCC
5' DYS 6	5'-5n3	intron 1 m.	70	96-118	114-116	60 PT	F TTCAGTTTCTCTCGGTGTTCCT R TACACCTGCACATGTGATGAAA
5' DYS 7*	5'-5n4	intron 4-5	64	133-159	133-133	60 PT	F GTCAGAACTTTGTACCTGTC R GAAGGGAAAATGATGAATAAAACT
STR07A	DXS 206	22 kb 3' e 7	68	218-235	227-227	55 PT	F TTCTGGTTTTCTGGTCTG R GAATCAATCTCTCTGTCAAG
STR07B	DXS 206	23 kb 3' e 7	44	227-241	233-233	55 PT	F AGCTATTATCTGAGAAAGTC R TGAGGTGAATTTATTAAGGG
5' DYS 5	5'-7n4	intron 25-28	52	163-169	164-166	60 PT	F GTGAAGCTACAAAAATATTAGAG R CAACAATATCTCACCATACTTG
STR 44A	DXS 1238	13,8 kb 3' e 44	85	174-204	180-190	55 PT	F TCCAACATTGGAAATCACATTTCAA R TCATCACAAATAGATGTTTCACAG
IVS44A*	IVS44SK21	50 kb 3' e 44	87	158-183	165-175	60 AT	F GGTGGTCTGAACAGCTCCAG R GCTCAGTGAATGAATAGCACAC

IVS44B	IVS44SK12	50 kb 3' e 44	38	177–185	181–181	60 AT	F CCTCAGTGAATGAATAGCACAC R TTGGTGAACAGCTCCAGAAGG
DXS1219	AFM297yd1	intron 44	54	230–246	230–244	55 PT	F TTAATGTTTCANCCAGGTAAAT R GATCACTCCAAAGGATAGATTGT
STR 44	DXS 269	40 kb 5' e 45	59	216–241	220–220	55 PT	F ACAGAAGGGGAGGTACAGCA R GCCACAACATATGCTTGAAAGA
STR 45	DXS 1237	1,2 kb 3' e 45	87	156–184	–	55 PT	F GAGGCTATAATTCTTTAACTTTGGC R CTCTTTCCCTCTTTATTTCATGTTAC
DXS997	AFM217xa5	9 kb 5' e 49	65	109–117	109–109	60 PT	F GTTTTCAGTTTCCTGGGT R TGGCTTTATTTTAAAGAGGAC
STR 49*	DXS 1236	intron 49	91	227–257	227–251	60 PT	F CGTTTACCAGCTCAAATCTCAAC R CATATGATACGATTCGTGTTTTGC
STR 50	DXS 1235	intron 50	70	233–251	241–241	60 PT	F AAGGTTCCCTCCAGTAACAGATTTGG R TATGCTACATAGTATGTCCTCAGAC
DXS 1067	AFM234vg7	5,5 kb 5' e 51	64	214–230	222–222	55 PT	F CCTCCAGTAACAGATTTGGGTG R TATGTCCTCAGACTATTCAGATGCC
DXS 1036	AFM072zh3	intron 51	62	145–151	149–149	60 AT	F TGCAGTTTATTATGTTTCCACG R GCCATTGATAAGTGCCAGAT
DXS 1241	DMD.PCR7	intron 55–57	51	245–255	–	60 PT	F ATAACTTACCCAAGTCATGT R TGTCTGTCTTCAGTTATATG
DXS 1214*	AFM283wg9	intron 62–63	79	210–220	216–216	60 PT	F TAGAACCCAAATGACAACCA R AAGATAGCAGGCAACAATAAGA
3' DYS 3	3'–19n8	3' e 63	58	140–150	145–147	60 PT	F AGCCCCATTCTGTACATCAAAT R AACGACTTCCCCACTCTGT
DXS 992	AFM184xg5	> 20 kb 3' e 79	87	201–211	202–211	65 PT	F AAGAATGGGACTCCATTTC R GCTTATCCACTGGGACAGAAAAG
DXS 1202*	AFM260ye5	Xp22.12	80	265–285	281–283	60 PT	F CCCAGCACAGGTGTCA R GGGTTGTTGCTCCAAAG
DXS 989*	AFM135xe7	Xp22	79	167–203	181–183	60 AT	F AGAAGATAGATATACCCACTCACC R ACATAAGAAATAAAATGGCTGC

\*Markers initially used in new families. PT, Promega Taq PCR buffer (Promega); AT, Ampli Taq PCR buffer (ABI); e, exon; b, brain transcript (Dp427b); m, muscle transcript (Dp427m).

8. 10% sodium dodecyl sulfate (SDS) (Life Technologies).
9. 10X SLM loading mix: 40% sucrose, 1% SDS, 100 mM EDTA, 100 mM Tris-HCl pH 7.5, 2.5 mg/mL bromophenol blue.
10. 10X TBE (Life Technologies).

## 2.2. Quantitative Multiplex PCR

In addition to the materials listed for the multiplex PCR, the following materials are needed:

1. AG 501-X8 Resin, molecular biology-grade (BioRad).
2. Formamide, deionized (Amresco, Lucron Bioproducts).
3. Deionized formamide. Check the pH—this should be above 8.0. Perform a deionization step with resin if the pH is lower. Add 1 g of AG 501-X8 resin to 25 mL formamide, mix for 10 min, and check the pH (using a pH dipstick). Aliquot the formamide (without pipetting the resin) into 500- $\mu$ L aliquots and store at  $-20^{\circ}\text{C}$  for a maximum of 1 yr.
4. GeneScan 1000 ROX size standard (Applied Biosystems [ABI]).
5. 3 M NaAc pH 4.6 (ABI).
6. 6% sequencing mix (Life Technologies).
7. Slab gel-loading mix: dissolve 500 mg Dextran Blue in 100 mL of deionized formamide and store at  $4^{\circ}\text{C}$  for a maximum of 2 yr.

## 2.3. Multiplex DGGE

1. Ficoll 400 (Amersham Pharmacia Biotech).
2. Deionized formamide (Amresco, Lucron Bioproducts).
3. 40% Acryl amide Bisacryl solution 37.5:1 (BioRad).
4. 50X TAE buffer (2 M Tris, 1 M NaAc.3H<sub>2</sub>O, 50 mM EDTA, pH 8.0), dissolve 484.5 g Tris, 272 g NaAc.3H<sub>2</sub>O in 37.3 g EDTA in 1 L of MilliQ water. Adjust the pH to 8.0 with acetic acid, and adjust the volume to 2 L with MilliQ water. Autoclave this solution and store at room temperature for a maximum of 1 yr.
5. 9% PAA, 80% UF (9% acrylamide, 32% formamide, 5.6 M urea in 0.5X TAE buffer), dissolve 210 g ureum in 6.3 mL 50X TAE, 140 mL 40% acrylamide/*bis* solution (37.5:1) and 200 mL deionized formamide and add MilliQ water to 625 mL. Store this solution protected from light at  $4^{\circ}\text{C}$  for a maximum of 3 mo.
6. 9% PAA, 0% UF (9% acrylamide in 0.5X TAE buffer), mix 225 mL 40% acrylamide/*bis*acryl solution (37.5:1) and 10 mL 50X TAE and add MilliQ water to 1 L. Store this solution at  $4^{\circ}\text{C}$  for a maximum of 1 yr.
7. 10% ammonium persulfate (AMPS), dissolve 10 g AMPS in MilliQ water and add MilliQ water to 100 mL. Aliquot this solution and store at  $-20^{\circ}\text{C}$  for a maximum of 1 yr or at  $4^{\circ}\text{C}$  for a maximum of 1 mo.
8. 6X polyacrylamide gel electrophoresis (PAGE) loading buffer (0.25% bromophenol blue, 0.25% xylene Cyanol, 15% Ficoll); store at room temperature for a maximum of 2 yr.
9. 10X PCR buffer: 100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% Triton X-100 (Promega).

## 2.4. STR Analysis

In addition to the materials listed for the multiplex PCR, the following materials are needed:

1. 2 mM dNTPs: prepare a mix containing 2 mM dATP, dCTP, dGTP, and dTTP in autoclaved MilliQ water and aliquot this solution. Store at  $-20^{\circ}\text{C}$  for a maximum of 1 yr.
2. 10X PCR buffer (PT): 100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% Triton X-100, and 15 mM  $\text{MgCl}_2$  (Promega).
3. 10X AmpliTaq PCR buffer (AT): 100 mM Tris-HCl pH 8.3, 500 mM KCl, and 15 mM  $\text{MgCl}_2$  (ABI).
4. GeneScan 500 TAMRA size standard (ABI).

## 3. Methods

### 3.1. Multiplex PCR for Deletion Detection

To ensure consistent quality and minimize batch-to-batch variation of the kits and too many freeze/thaw cycles of the primer stocks, it is recommended to prepare the kits in larger batches. Use the protocol described here to prepare multiplex kits for the detection of deletions in patients (7,8). Only the DNA template and Taq polymerase must be added to these kits prior to the PCR reaction. During batch preparation, positive and normal controls are tested to validate and ensure good quality of the kits.

#### 3.1.1. Preparation in Batches of Multiplex PCR Kits

1. Prepare the master mix for each kit according to **Tables 1, 2, and 8** (e.g., for 200 reactions; *see Note 1*). Final concentrations of the mix for the deletion detection kits are 1X multiplex PCR buffer, 10% DMSO, 1.5 mM dNTPs, 170 ng/ $\mu\text{L}$  BSA, and 0.5  $\mu\text{M}$  of each primer.
2. To test the master mix, perform a PCR (*see Subheading 3.1.2.*) with DNA of patients with different deletions (positive controls), normal controls, and a no-DNA control (*see Note 2*). To test six samples and a no-DNA control, pipet 189  $\mu\text{L}$  of the prepared master mix in an Eppendorf tube and add 3.2  $\mu\text{L}$  Taq polymerase. Mix this solution well, and pipet 24  $\mu\text{L}$  into PCR tubes. Continue with **step 3 of Subheading 3.1.2.** Store the master mix at  $4^{\circ}\text{C}$  during the test.
3. Check the correct band pattern on an agarose gel (*see Subheading 3.1.3.*). When results are as expected, aliquot the kit (e.g., 200- $\mu\text{L}$  aliquots) and store at  $-80^{\circ}\text{C}$  for a maximum of 2 yr (*see Note 3*).

#### 3.1.2. Amplification

1. Defrost an aliquot of the multiplex kit on ice. Pool aliquots if more samples must be analyzed.

**Table 8**  
**Pipetting Scheme for Batch Preparation of Multiplex**  
**PCR Deletion Screening Kits**

	Volumes per reaction		
	Deletion screening kits	5' quantitative	3' quantitative
Autoclaved MilliQ water	13.275 $\mu$ L	14.255	14.215
5* PCR buffer	5 $\mu$ L	5 $\mu$ L	5 $\mu$ L
DMSO	2.5 $\mu$ L	–	–
25 mM dNTPs	1.5 $\mu$ L	0.5	0.5
BSA 10.2 mg/mL	0.425 $\mu$ L	0.425	0.425
Forward primer (250 pmol/ $\mu$ L)	9 $\times$ 0.05 $\mu$ L	13 $\times$ 0.02	14 $\times$ 0.02
Reverse primer (250 pmol/ $\mu$ L)	9 $\times$ 0.05 $\mu$ L	13 $\times$ 0.02	14 $\times$ 0.02
End volume	23.6	20.7	20.7

2. Add 2 U of Taq polymerase per 24  $\mu$ L of reaction mix, mix well, and pipet 24  $\mu$ L into PCR tubes.
3. Add 1  $\mu$ L of genomic DNA (200–500 ng) to the tube (*see Note 4*). For the no-DNA control, just use 24  $\mu$ L of master mix.
4. Place the PCR tray in a preheated thermal cycler, set at 95°C (hot start). Perform an initial denaturation of 5 min at 95°C followed by 25 cycles of 94°C for 30 s, 53°C for 30 s, and 65°C for 4 min, with a final extension period of 5 min at 65°C. Cool down to 4°C after the PCR has finished.

### 3.1.3. Gel Electrophoresis of the PCR Products

1. Prepare a 2.5% Nusieve and 0.5% multipurpose agarose gel in 1X TBE buffer with 0.2  $\mu$ g/mL ethidium bromide (*see Note 5*).
2. Add 2.7  $\mu$ L of 10X SLM loading mix to the PCR reaction. Heat the 10X SLM before usage in hot water if a precipitation of SDS is visible.
3. Place the Nusieve gel in an electrophoresis tank containing 1X TBE running buffer with 0.2  $\mu$ g/mL ethidium bromide. We use the Sub-Cell GT Agarose Gel Electrophoresis Systems from BioRad.
4. Load 25  $\mu$ L of the PCR reaction on gel, including the no-DNA template reaction. Use a clean tip for each loading. Also, load a suitable size standard (e.g.,  $\Phi$ X-174 *Hae*III fragments).
5. Electrophorese the gel for 2–3 h at 100–150 V. The bromophenol blue dye must migrate at least 10 cm into the gel for a good separation.
6. Prepare an image of the gel using an imaging system. Check for absence of product formation in the no-DNA control. Score the deletions of exons using



**Tables 1 and 2** (see **Note 6**). In **Fig. 2**, a typical gel image is presented of both multiplex PCRs on patient and normal control samples.

### **3.2. Quantitative Multiplex PCR for Deletion and Duplication Detection**

Two quantitative multiplex kits are used for deletion and duplication detection in both carriers and patients (**9**). For these kits, it is advisable to use a system as described for the multiplex kits, and prepare batches of both 5' and 3' multiplex kits.

#### **3.2.1. Preparation in Batches of Quantitative Multiplex PCR Kits**

1. Prepare the master mix for each kit according to **Tables 3, 4, and 8** (e.g., 200 reactions; see **Note 1**). Final concentrations of the mix for the kits are 1X multiplex PCR buffer, 0.5 mM dNTPs, 170 ng/ $\mu$ L BSA, and 0.2  $\mu$ M of each primer.
2. Perform a PCR (see **Subheading 3.2.2.**) with DNA of carriers with different deletions or duplications (positive controls), normal controls, and a no-DNA control to test the master mix (see **Note 2**). Pipet 248  $\mu$ L of the prepared master mix in a microcentrifuge tube to test 11 samples, and add 3.6  $\mu$ L Taq polymerase. Mix this solution well, and pipet 21  $\mu$ L into PCR tubes. Continue with **step 3** of **Subheading 3.2.2.** Store the master mix on ice at 4°C during the test and protect the tubes from direct light (the fluorescent labels are light-sensitive).
3. Check the correct peak pattern by electrophoresis, using an ABI Prism Analyser (see **Subheading 3.2.3.**). After the electrophoresis, calculate the DQ values of the positive controls (see **Subheading 3.2.4.**). When results are as expected, aliquot the kit (e.g., 200  $\mu$ L aliquots) and store at -80°C for a maximum of 2 yr.

#### **3.2.2. Amplification of Quantitative Multiplex PCR**

1. Defrost an aliquot of the multiplex kit on ice. Pool aliquots if more samples must be analyzed.
2. Add 1.5 U of Taq Polymerase per 21  $\mu$ L of reaction mix, mix well, and pipet 21  $\mu$ L into PCR tubes.
3. Add 4  $\mu$ L of genomic DNA (50 ng/ $\mu$ L) to the tube (see **Note 7**). With every series (5' and 3' kit), two normal control DNA samples are also amplified with the samples to be tested simultaneously (see **Note 8**).
4. Place the PCR tray in the preheated thermal cycler, and set at 95°C (hot start). Perform an initial denaturation of 6 min at 94°C followed by 18 cycles of 94°C for 48 s, 58°C for 48 s, and 70°C for 3 min, with a final extension period of 7 min at 70°C. Cool down to 4°C after the PCR has finished.

#### **3.2.3. Preparation of Samples Prior to Electrophoresis**

1. To avoid primer interference during electrophoresis on a capillary electrophoresis unit, it is advisable to concentrate the sample and remove some of the primers

- by precipitation (*see Note 9*). Add 160  $\mu\text{L}$  of 2-propanol and 20  $\mu\text{L}$  3 M NaAc pH 4.6 to a microcentrifuge tube and mix on a vortex for a few seconds.
2. Add 20  $\mu\text{L}$  of the PCR product and mix again on the vortex.
  3. Leave for 10 min at room temperature.
  4. Centrifuge for 20 min at maximum speed in a microcentrifuge.
  5. Localize the pellet and carefully aspirate the supernatant (*see Note 10*).
  6. Wash the pellet with 250  $\mu\text{L}$  of 70% ethanol, vortex briefly, and centrifuge for 10 min at maximum speed.
  7. Aspirate the supernatant and leave the open tubes for 10 min at room temperature or 1 min at 90°C in a heating block to dry the pellet (*see Note 11*).
  8. Add 20  $\mu\text{L}$  of deionized formamide and dissolve the pellet by vortexing. Prepare a master mix of 5  $\mu\text{L}$  deionized formamide and 0.5  $\mu\text{L}$  of ROX-1000 size standard per sample and aliquot 5.5  $\mu\text{L}$ . Add 10  $\mu\text{L}$  of the sample to the formamide/size standard mix (*see Note 12*).
  9. Denature the samples for 3 min at 95°C and then cool on ice.

#### 3.2.4. Electrophoresis of the Samples and Data Analysis (*see Note 13*)

1. For analysis on an ABI Prism 310, use the POP4 polymer and the GS STR POP4 (1 mL) module. Change the Syringe Pump Time from 150–300 s and the Collection time from 24–35 min. For data collection of 6-FAM, HEX, and ROX labels, use filter set A or C. Samples are run on a 47-cm capillary.
2. Analyze the raw data in GeneScan Analysis. Change the default analysis parameters for analysis range 3,500–10,000 data points, size call range 100–600 bp and minimum peak half-width 9 (*see Note 14*).
3. The Genotyper software is used for automated data analysis. With this software, the peaks are labeled with the exon name, the length of the fragment, and the peak area (*see Fig. 3*). From the Genotyper-analyzed data, a table is then generated and exported so the data can be used for DQ value calculations. To do this, define a template file in Genotyper (*see Note 15*). Import the GeneScan analyzed data for the 5' kit and the 3' kit in the appropriate template file and run the label peaks option. Append all the data to the table and export the table to a file.
4. To calculate the DQ values for all samples in an automated fashion, a spreadsheet template file is created (*see Note 16 and Fig. 4*). Open both the table file exported from Genotyper and the spreadsheet template file, and import the peak areas of two control samples and the sample of interest using a copy and paste function.
5. Determine the presence of a deletion or duplication in the analyzed samples by comparison of the calculated DQ values to the ranges presented in **Table 5**. To score a deletion or duplication, all DQ values for an exon should consistently be in the deletion or duplication range (*see Note 17*).

### 3.3. Multiplex DGGE

To amplify all exons of the dystrophin gene 95, amplicons must be generated through PCR. For most optimal mutation detection, 15 exons were split

into two PCR fragments. For primer sequences of all amplicons and length of PCR products, *see* **Table 6**. Although multiplex PCRs can be performed it is advised to amplify all amplicons separately and combine the PCR products to get the different multiplex DGGEs as shown in **Table 9**.

### 3.3.1. Amplification of DGGE Fragments and Sample Preparation

1. For each amplicon, set up a PCR in a 30- $\mu$ L end volume containing 1X PCR buffer, 250  $\mu$ M dNTPs, 0.25  $\mu$ M primers, 200 ng template DNA, and 1.2 U Taq polymerase (*see* **Note 1**). For some amplicons, the standard concentration of MgCl<sub>2</sub> is increased from 1.5 mM to 3 mM (amplicon 3A and 3B), or 8.5 mM (amplicon 13B and 61A).
2. Place the PCR tray in the preheated thermal cycler, set at 95°C (hot start). Perform an initial denaturation of 5 min at 96°C followed by:

5 cycles 30 s 94°C, 30 s 53°C and 1 min 72°C

5 cycles 30 s 94°C, 30 s 50°C and 1 min 72°C

3 cycles 30 s 94°C, 30 s 48°C and 1 min 72°C

15 cycles 30 s 94°C, 30 s 50°C and 1 min 72°C

13 cycles 30 s 94°C, 30 s 48°C and 1 min 72°C

5 min 72°C

Cool down to 4°C after the PCR has finished.

3. Test 5  $\mu$ L of the PCR products on a 2.5% agarose gel (*see* **Note 2**).
4. After a good amplification, 7  $\mu$ L of each PCR product is used for DGGE. This can be adjusted if amplification is weaker. For patient samples, pool the PCR products of two patients per multiplex according to **Table 9**. Also, combine the PCR products per multiplex for carriers. After pooling the samples, perform a heteroduplex step of 10 min at 96°C and 15 min at 48°C (*see* **Note 3**).
5. Add 1  $\mu$ L of 6X PAGE loading dye to every 5  $\mu$ L of PCR product.

### 3.3.2. Preparation of the DGGE Gradient Gel (*see* **Note 4**)

1. Use glass plates thoroughly cleaned with 70% ethanol, and assemble the gel cassette using a casting stand according to the manufacturer's guidelines.
2. Prepare the high- and low-density gel solution by pipetting the amounts needed into two 50-mL tubes (*see* **Table 10**). In this setup, a 25-mL volume will just fill the plates.
3. Put a stirring magnet in each column of the gradient mixer and place it on a magnetic stirrer that is held above the casting stand. Close the connection between the two columns and close the outlet tube. Place the end of the outlet tube between the glass plates, preferably at the side. Turn on the magnetic stirrer and check that the magnet in the column with the outlet tube is stirring in the middle of the column.

**Table 9**  
**Composition of DGGE Multiplex and Gradient Gel**

Multiplex	Amplicons	Gel gradient %
1	1, 2, 6A and 6B	15–55
2	27, 28, 33 and 35	15–55
3	32, 46 and 47	15–55
4	13A, 44 and 70	15–55
5	12, 13B, 18B and 19	20–60
6	31, 34, 45 and 49	20–60
7	53A, 58 and 72	20–60
8	66, 74, 77 and 78	20–60
9	54B, 67, 73 and 79	20–60
10	26A, 29A and 43A	20–60
11	15, 29B and 30	20–60
12	16, 48 and 63	20–60
13	20B, 43B and 50	20–60
14	36, 38 and 65	20–60
15	5, 23A and 69	20–60
16	7, 18A and 39	20–60
17	53B, 55 and 68B	20–60
18	11, 22 and 25	20–60
19	10, 24 and 51B	20–60
20	4, 8 and 17	20–60
21	3B, 9 and 21	20–60
22	42, 57 and 64	20–60
23	20A, 51A and 76	20–60
24	40 and 52	20–60
25	23B, 37 and 68A	35–75
26	41A, 61A and 61B	35–75
27	41B, 54A and 62	35–75
28	56, 60 and 71	35–75
29	3A and 75B	35–75
30	75A	35–75
31	59	20–60
32	26B	20–60
33	14	15–55 (62°C)

4. Add 100  $\mu$ L 10% AMPS to both density solutions, mix, and add 5  $\mu$ L TEMED to each solution, and gently mix again. Pour the high-density solution into the column with the outlet tube. Open the connection between the two columns shortly to remove air bubbles. Pour the low-density solution into the other column and open

the outlet tube. As soon as the solution is running through the outlet tube, open the connection between the columns. Fill the sandwich until all the solution is used. Let the gel polymerize for about 60 min (*see Note 5*).

5. After polymerization, wash the gel with MilliQ water. Remove any excess of water with a piece of filter paper, and position the comb between the glass plates, about 2 mm above the gel surface.
6. Prepare the stacking gel by adding 50  $\mu\text{L}$  10% AMPS and 2.5  $\mu\text{L}$  TEMED to 3.5 mL 9% PAA, 0% UF solution. Pour the solution on top of the gradient gel and leave to polymerize for 15–30 min.

### 3.3.3. Electrophoresis and Visualization of Bands

1. Gels are electrophoresed in 0.5X TAE buffer. Preheat the buffer to 58°C (or 62°C for exon 14 only) (*see Note 6*).
2. Remove the comb gently and rinse the slots directly with MilliQ water or running buffer to remove unpolymerized solution.
3. Assemble the gel sandwich onto the core of the electrophoresis unit and place the complete system into the buffer chamber, according to the manufacturer's guidelines.
4. Load the samples using gel-saver tips, turn on the power, heating, and pump, and electrophorese at 80 V for 16 h.
5. After electrophoresis has been completed, take out the gel and disassemble the system. Use one of the two glass plates to support the gel during staining and gently shake the gel for 5–10 min in  $\pm$  250 mL MilliQ water containing 10  $\mu\text{L}$  ethidium bromide (*see Note 7*).
6. Prepare an image of the gel using an Imaging system. Score the frame-shifts present on the gel and determine whether the positive controls are visible. In **Fig. 5**, a typical gel image is presented of a multiplex DGGE (*see Note 8*).

## 3.4. STR Analysis

In order to produce a valid haplotype analysis, it is essential that key individuals are used in the experiment to be able to detect the at-risk haplotype and follow its segregation. For new families, use the set of 8 markers presented in **Table 7**, or select the appropriate markers from the list that will produce enough information. The protocol described here has been optimized for use of fluorescently labeled forward primers and electrophoresis on a DNA sequence detection system (ABI Prism 310 DNA Analyzer, Applied Biosystems).

1. For each STR marker, set up a PCR in a 25- $\mu\text{L}$  end volume containing 1X PCR buffer, 200  $\mu\text{M}$  dNTPs, 0.5  $\mu\text{M}$  primers, 200 ng template DNA, and 1 U Taq polymerase (*see Note 1*).
2. Place the PCR tray in the preheated thermal cycler, and set at 95°C (hot start). Perform an initial denaturation of 5 min at 95°C followed by 30 cycles of 94°C for 30 s, 55/60/65°C for 30 s, and 72°C for 30 s with a final extension period of 5 min at 72°C. Cool down to 4°C after the PCR has finished.

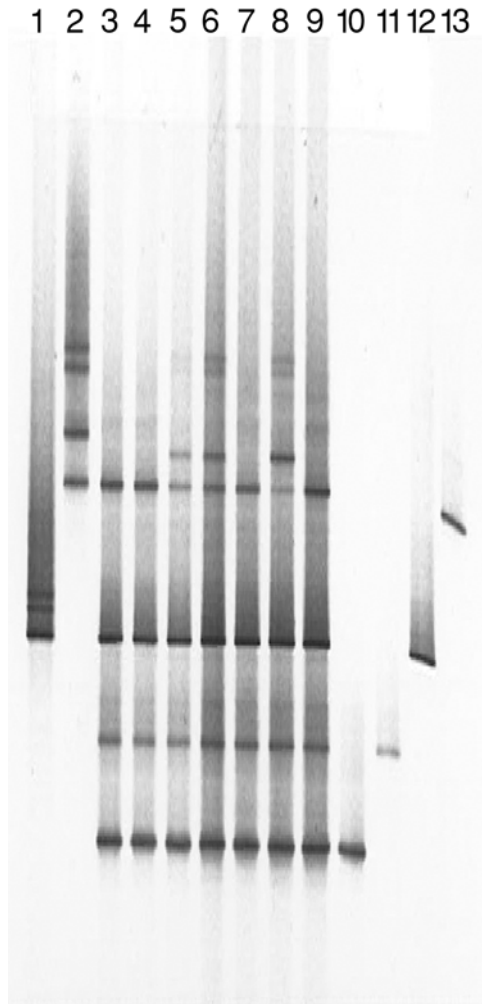


Fig. 5. Gel image of DGGE multiplex 8, exons 66, 74, 76, and 78. Lane 1 and 2: Positive control for exon 74 (Glu3515X) and exon 66 (Gly3189-3217delfsX3229) respectively. Lanes 3 to 5: Carrier samples. Lanes 6 to 9 represent patient samples, pooled two by two. Lanes 10 to 13: Normal controls for each exon loaded separately—exons 78, 76, 74, and 66, respectively. Note that the common polymorphism (9857 + 15C > T) is detected in exon 66, which clearly differs from the band shift pattern of the exon 66 mutation (lane 2).

3. Check for correct amplification of the marker by testing 5  $\mu$ L of the PCR product on a 2.5% agarose gel. Prepare a correct dilution of the PCR products and pool appropriate STR markers of one DNA sample (*see Note 2*).

**Table 10**  
**Preparation of Denaturing Acrylamide Solutions**

% UF	0% UF (mL)	80% UF (mL)
15	10.2	2.3
20	9.4	3.1
35	7.0	5.5
55	3.9	8.6
60	3.1	9.4
75	0.8	11.7

UF, Urea/formamide mix

4. Prepare a master mix of 10  $\mu$ L deionized formamide and 0.5  $\mu$ L of TAMRA-500 size standard per sample and aliquot 10.5  $\mu$ L. Add 1–5  $\mu$ L of the diluted PCR product to the formamide/size standard mix (*see Note 3*).
5. Denature the samples for 3 min at 95°C and cool on ice afterwards.
6. Electrophorese the samples using standard settings, for the ABI 310 module GS POP4 STR (*see Note 4*).
7. Analyze the data using the appropriate software—for ABI runs, use GeneScan Analysis and Genotyper; for ALF runs, use Fragment Manager (*see Note 5*).

## 4. Notes

### 4.1. Multiplex PCR

1. To avoid PCR contamination, physically separate pre-PCR and post-PCR activities. Preparation of template DNA and setting up the PCR should be performed in a pre-PCR laboratory, whereas PCR cycling and handling of all PCR products is performed in a post-PCR laboratory. **Caution:** Never move laboratory equipment and durables from the post-PCR laboratory to the pre-PCR lab without cleaning thoroughly.
2. To validate and ensure the quality of the kits, control samples must be tested on the master mix prior to the preparation of aliquots. We use two no-deletion controls and four deletion controls with deletions throughout the gene for the deletion kits and DNA samples of female controls of three no deletion, five deletion, and two duplication control samples for the quantitative multiplex kits.
3. If intensities of some amplicons are low compared to the other amplicons, one may increase the yield of these amplicons somewhat by doubling the primer concentration. Storage of primer stocks for longer periods and repeated freeze/thaw cycles may result in poor amplification. For these amplicons, it is advisable to order new primer batches of both forward and reverse primer, and check correct synthesis by performing a test PCR of this individual amplicon. Storage of primer stocks at –80°C in TE-4 buffer is recommended.

4. When using kits for scanning for deletions, or duplications (quantitative multiplex), no positive control is used, since the kits were validated during the preparation. A normal control is not necessary if three or more nonrelated DNA samples are tested in the scanning, since the chance of finding the exact same mutation in all samples tested is negligible. If the techniques are used for confirmation or exclusion of the presence of a known mutation (e.g., carrier determination or prenatal diagnosis), positive and negative controls are used throughout the experiment. If different mutations are examined in one experiment, then for each type of mutation (deletion or duplication), one positive control sample is used. Furthermore, in our laboratory we have chosen to isolate the DNA in duplicate and use the second DNA sample to confirm a mutation found during the scanning, and use both samples simultaneously for carrier detection.
5. For a 150-mL gel, approx  $15 \times 12 \times 0.75$  cm, add 3.75 g Nusieve with 0.75 g Multipurpose agarose to 1X TBE buffer. Mix well to yield a homogeneous suspension before heating in a microwave. Slowly heat up the solution to prevent foam formation. When the solution is homogeneous, cool down to 50–60°C, add the ethidium bromide, and cast the gel. Leave the gel for 1 h to solidify.
6. Use the data only if the no-DNA control is indeed negative and all of the expected amplicons are observed at least once throughout the gel. Use the data of both kits to confirm deletions of several exons—e.g., an absence of amplicons for exon 45 and 48 observed with kit 1 must coincide with an absence of exon 47 observed with kit 2. If a deletion of only one exon is observed, perform Southern blot analysis to confirm this deletion or use a different primer set for this exon in the PCR reaction to rule out rare polymorphisms in the primer sites that might prevent annealing of the primer and amplification. For alternative primer sequences, see the “Leiden Muscular Dystrophy pages” (<http://www.dmd.nl/exonprim.html>), or make use of the DGGE primers described in **Table 6**.
7. Quantify the template DNA by spectrophotometrical measurement at 260 nm or electrophoresis of a dilution on an agarose gel and comparison to a quantitative DNA ladder or lambda concentration range. Prepare dilutions of 50 ng/ $\mu$ L in autoclaved MilliQ water. In our experience, it is best to use freshly prepared dilutions, dissolved overnight at 4°C. Sometimes older DNA samples that have been stored for many years may cause problems in the quantitative PCR. If this is observed, it is best to purify some of the DNA using any of the known methods for DNA purification or isolation (e.g., DNA mini kit [Qiagen] or phenol extraction).
8. Use two DNA samples of female normal controls found to be negative for a deletion or duplication in the dystrophin gene. The average peak area for each exon of these controls is used in the calculations for the DQ values of the samples to be tested.
9. Samples that are run on a capillary system without precipitation result in poor signal intensities and extra nonspecific peaks in the electropherogram because of an overload of the column with labeled primers. Because of the electrokinetic injection,



- shorter fragments and primers are preferentially injected into the capillary. Also, on slab gel systems, the excess of labeled primers can cause background problems.
10. The pellet is easily removed together with the supernatant. Thus, aspirate the supernatant with a capillary glass pipet, prepared from glass Pasteur pipets to avoid accidental removal of the pellet. Always mark the orientation of the tubes in the centrifuge, and after centrifugation, a clear pellet should be visible.
  11. To ensure that the pellet will dissolve in formamide, make sure that no ethanol residues are present, but also take care to prevent the pellet from becoming too dry.
  12. When using a slab gel system for the electrophoresis, use the slab gel-loading mix instead of the deionized formamide.
  13. The protocol described here is for electrophoresis using an ABI Prism 310 capillary system. However, other systems can be used. Use a 5–6% denaturing polyacrylamide gel in 1X TBE buffer for a slab gel system and load 6–10  $\mu\text{L}$  of the sample on gel. Perform an overnight run (500 min) at constant power of 30–45 W. Use the appropriate internal size standard.
  14. The ROX 1000 size standard peak sizing should be in the range of 108–677 bp. If this is not the case, define a new size standard using the raw data of the size standard for this sample and reanalyze. Check for split peaks, and if necessary, change the minimal half peak width to 10 or 11, or alternatively, use the peak area of both peaks in the DQ value calculations. See the GeneScan user's manual for details on how to operate the software. Intensities of the raw data peaks should be in the range of 1000–2000 fluorescent units. Use an increased injection time (ABI 310) or load more sample on gel if intensities are too low. Dilute the sample if the signals are saturated and DQ values are out of range.
  15. In a template Genotyper file, one for the 5' kit and for the 3' kit, create a category for each exon in which you define a basepair range of the average basepair length + and –5 bp (see **Tables 3** and **4**), select the color of the peak (blue for 6-FAM, green for HEX labels in filter set C) and select "Highest peak." Since a 2 bp-deletion polymorphism is detected in exon 43, select "Highest 2 peaks" for this category. Also use this setting if split peaks are observed frequently for a certain exon. Set up a table in which the sample names, the category names, the fragment lengths, and the peak areas are presented. See the GenoTyper user's manual for details on how to operate the software.
  16. Set up a spreadsheet template file that calculates the DQ values as shown in **Fig. 4**. For the calculation of DQ values, use the formula. Set up the spreadsheet so that the mean control peak areas are calculated automatically when the data is imported into the spreadsheet.
  17. Although a small overlap is sometimes seen between some of the DQ values in the normal and those in the deletion/duplication range, especially for samples with poorer amplification signals, a deletion or duplication should be easy to score. If only a one-exon deletion or duplication is observed, always confirm the results using a different, non-overlapping primer set for this exon in the quantitative multiplex or using another technique such as Southern blotting or RT-PCR (see also **Note 6**).

## 4.2. Multiplex DGGE

1. If a robotic system is available to perform the pipetting steps, it is best to prepare a master mix containing all components without primers and template DNA. Start with dispensing of the master mix in the PCR plate, followed by the addition of primers, and end with template DNA. Alternatively, prepare a PCR mix including the primers of a single amplicon, dispense in the PCR plate, and add the template DNA.
2. If large series are used, only test the PCR products of a few samples per fragment for correct amplification.
3. Use equal volumes of PCR products for pooling, adjust the ratio if for one of the amplicons weaker amplification is observed. The heteroduplex step is to enhance heteroduplex formation of the different DNA strands, and thereby easier detection of band shifts on DGGE gel. To allow heteroduplex formation, PCR products of two patients must be pooled. For carrier detection, this is not necessary or advisable. The template DNA of two patients can be mixed prior to amplification as an alternative to the pooling of PCR products afterwards. However, this method is not advised, since poor amplification of one or both templates is not detected in such a system. On each DGGE gel, load a positive control for every amplicon if available. It is also advisable to load each amplicon from a multiplex DGGE separately to allow easy detection of the corresponding bands in the multiplex. To combine these two control settings, use the positive control—or in the absence thereof, a normal control—and load these in separate lanes (*see Fig. 5*).
4. For the DGGE analysis we use the Dcode Universal Mutation Detection System from BioRad, and the protocol described is for using this system. However, any commercially available or homemade system for vertical PAGE in a temperature-controlled electrophoresis tank should work well. The type of gradient former is very important for the formation of a correct gradient. We use the BioRad gradient former model 385, placed approx 35 cm above the casting stand. Gel formats are 20 × 16 cm, with spacers of 1-mm thickness. Use a comb that allows loading of 40 μL of sample.
5. Check whether the low-density solution is mixing with the high-density solution. If not, there may be an air bubble in the connection between the two columns. Ensure an even flow of both solutions through the gradient former. Clean the gradient former and tubing directly after use to prevent polymerization of polyacrylamide solution and clogging of the channels.
6. Replace the 0.5X running buffer for each run. Re-using the running buffer may affect the migration rate and band resolution. Increase the preheat temperature by 6°C, so that heating up the running buffer after the sample loading will take less time.
7. If a high background is observed, a destaining step of about 15–20 min in 250 mL MilliQ water can be introduced.
8. To determine whether band shifts are the result of the causative mutation or a polymorphism, fragments should always be checked using direct sequencing. Since polymorphisms are likely to be seen, it is best to complete the DGGE scan and afterwards sequence the most promising band shifts first. Since band patterns are

usually fairly specific for a sequence alteration, the more common polymorphisms are recognizable on the DGGE gel. It is not advisable to sequence the DGGE PCR product because this will often result in poor sequence data. Instead, use sequence primers containing unique universal sequence tails (M13) on both forward and reverse primers, and use these sequence tails as sequence primer. For details on sequence primers, *see* the “Leiden Muscular Dystrophy pages” (6).

### 4.3. STR-Analysis

1. Dilutions should be prepared for the template DNA and used for PCR instead of pipetting directly from the stock. Over amplification resulting from too much template DNA in the PCR reaction may lead to extra stutter band in the electropherogram or overall failure of the PCR. The PCR for the STR markers can be performed per STR marker, or in a multiplex assay if allele sizes are taken into consideration or different fluorescent labels are used.
2. After a good amplification, PCR products usually must be diluted 5–10 times prior to electrophoresis. To determine the correct dilution factor, perform the agarose gel electrophoresis. If not diluted correctly, signals become too high, which may result in false allele sizing or missing of heterozygotes during analysis and interpretation. It is important to use a reference control sample such as CEPH sample 134702 as an external control. Also, prepare equal dilutions of the no-DNA control samples, and use these samples in the complete procedure. If not amplified in a multiplex PCR, products of appropriate markers of one DNA sample can be pooled at this time.
3. Use the slab gel-loading mix instead of deionized formamide, and prepare a total loading volume of 10  $\mu$ L maximum for a slab gel system (ABI 377 Applied Biosystems: ALF, Amersham Pharmacia Biotech).
4. Use a 5–6% denaturing polyacrylamide gel in 1X TBE buffer for a slab gel system and load 6–10  $\mu$ L of the sample on gel. Perform a run of 300 min at constant power of 30–45 W. Use the appropriate internal size standard.
5. Stutter peaks are visible in the electropherogram for most STR markers. These peaks are more intense for some markers than for others. Also, peak patterns may differ but are relatively unique for a marker when comparing different runs. Peaks with the highest intensities represent the alleles. If alleles only differ by 1 or 2 bp, the two largest peaks usually represent both alleles, with the second peak as most intense, since this peak is formed by the smaller allele plus the stutter band of the larger allele.

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## Spinal Muscular Atrophy

Hans Scheffer

### 1. Introduction

The spinal muscular atrophies are a clinically and genetically heterogeneous group of neuromuscular disorders caused by degeneration of anterior horn cells. In proximal spinal muscular atrophy (SMA), the muscles of the extremities closest to the trunk are affected earlier and more severely. SMA is clinically classified into four types: acute or Werdnig-Hoffmann disease, intermediate, mild or Kugelberg-Welander disease, and adult (SMA types I, II, III, and IV, respectively) (*1,2*). The clinical criteria are summarized in **Table 1**. With an estimated incidence of  $\sim 1/10,000$  (*3–7*), and a carrier frequency of  $1/40–1/60$  (*8,9*), the SMAs are among the most frequent autosomal recessive hereditary disorders, and the incidence appears to be similar in different populations studied.

A SMA type IV has been described by Pearn (*6*). In this subtype, the clinical symptoms begin after the age of 30 yr in the majority of cases.

The Survival of Motor Neuron (SMN) gene exists as two homologs, at least one telomeric gene (SMN1) and in the vast majority of chromosomes at least one centromeric gene (SMN2) per chromosome in normal (non-carrier) individuals, although also chromosomes with an intact SMN1 gene exist that lack SMN2 (*10*). The two SMN genes (SMN1 and SMN2) are highly homologous, and contain only five base pair (bp) differences that occur within their 3' regions (*11*). These differences in exons 7 and 8 are used to distinguish SMN1 and SMN2 in DNA analysis. Rearrangements of the SMN1 gene appear to be directly involved in SMA, since exon(s) 7 (and 8) of SMN1 are undetectable in more than 95% of patients, regardless of their clinical type (*10,12–16*), either as a result of homozygous deletions, or because of the conversion of sequences of SMN1 into those of the SMN2 gene (*10,14–19*). The clinical phenotype of

**Table 1**  
**Criteria for Clinical Classification of SMA Patients**

Clinical subtype	Age of Onset (in months)	Motor milestones	Death (in years)
SMA type I	<6	Never sit unaided	<2
SMA type II	<18	Never walk unaided	>2
SMA type III	>18	Stand unaided	adult

SMA appears to be partly determined by the underlying mutational mechanism. Most cases of SMA type I result from homozygous gene-deletion events, whereas the milder forms of the disease, SMA types II and III, are more likely to result from gene conversion of SMN1 into SMN2; SMA type II from a gene conversion event in one allele in addition to a hemizygous deletion event in the other allele; in SMA type III from gene conversions in both alleles (*10,17–21*). As a result, patients suffering from the milder forms of SMA carry more copies of the SMN2 gene. However, when available, these data cannot presently be used to predict the clinical course of the disease.

### 1.1. Strategy

The absence of SMN1 exon 7 in SMA patients can be used as a diagnostic tool for SMA (*10,22*), with a sensitivity of approx 95% in clinically strictly defined SMA patients in most populations (**Fig. 1**). The remaining patients (SMA patients without a homozygous SMN1 exon 7 absence) are virtually all assumed to have a hemizygous SMN1 absence of at least exon 7, either by deletion or by conversion. A quantitative assay based on competitive amplification of SMN1 and SMN2 exons 7 to determine their gene-copy number is also described in this chapter (**Fig. 2**).

Confirmation of the clinical diagnosis or suspicion of SMA by the detection of homozygous SMN1 exon 7 (and exon 8) rearrangement is fast, inexpensive, and reliable. Best practice guidelines for the molecular analysis of SMA have been published (*23*).

#### 1.1.1. Direct Deletion/Gene Conversion Detection of the SMN1 Gene

The presence of a homozygous deletion of SMN exon 7 is consistent with a diagnosis of SMA because there are clinical grounds for suspecting SMA. If SMN1 exon 7 is not homozygously absent, a diagnosis of SMA is not ruled out, since approx 5% of cases are known to have no SMN1 deletion or gene conversion in both alleles. A proportion of those SMA patients will have a subtle mutation in one—or extremely rarely, in both—SMN1 alleles instead (*24*).



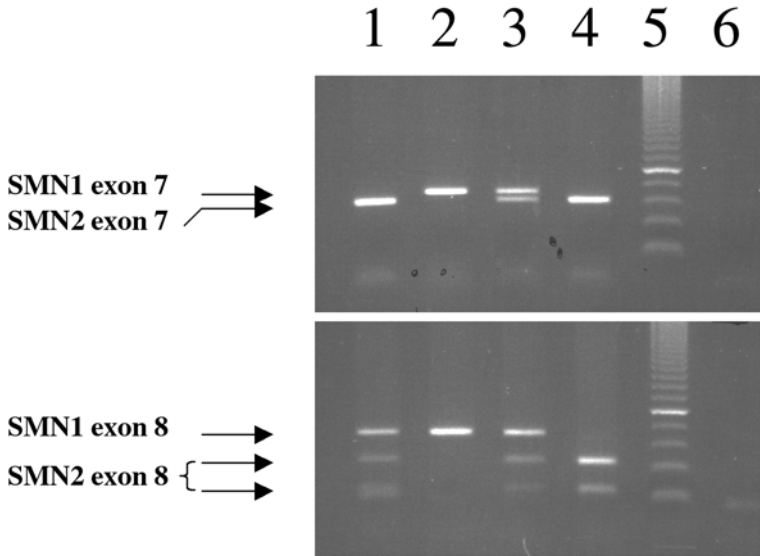


Fig. 1. Direct SMN1/2 homozygous absence of exon 7 and 8. The position of the different exons is indicated. Lanes 1 and 3, no homozygous absence of either SMN1 or 2 exons 7 or 8; Lane 2, homozygous absence of SMN2 exons 7 and 8 (not indicative of SMA); Lane 4, homozygous absence of SMN1 exons 7 and 8 (indicative of SMA); Lane 5, length marker; Lane 6, no template control (primer dimer bands can be seen).

The presence of a hemizygous SMN1 exon 7, identified by a quantitative determination of the number of SMN1 exon 7 copies, may provide further support for the diagnosis of SMA. Nevertheless, formal confirmation of the clinical diagnosis in these cases can only be obtained after identification of subtle SMN1 mutation(s).

## 1.2. Carrier Testing

### 1.2.1. Direct Carrier Testing

A second reason for referral is carrier detection by direct hemizygous SMN1 exon 7 analysis and/or indirect linkage analysis. Carrier detection by direct analysis is indicated in siblings and close relatives of SMA patients with a homozygous SMN1 exon 7 (and 8) absence, in parents of SMA patients who died, and in partners of known carriers without a positive family history for SMA. Indirect analysis can only be applied for family members of a patient, assuming that an adequate number of family members are available for analysis to establish linkage phase. However, direct analysis is strongly recommended in these cases.

## Hemizygous SMN Exon 7 Deletion Analysis

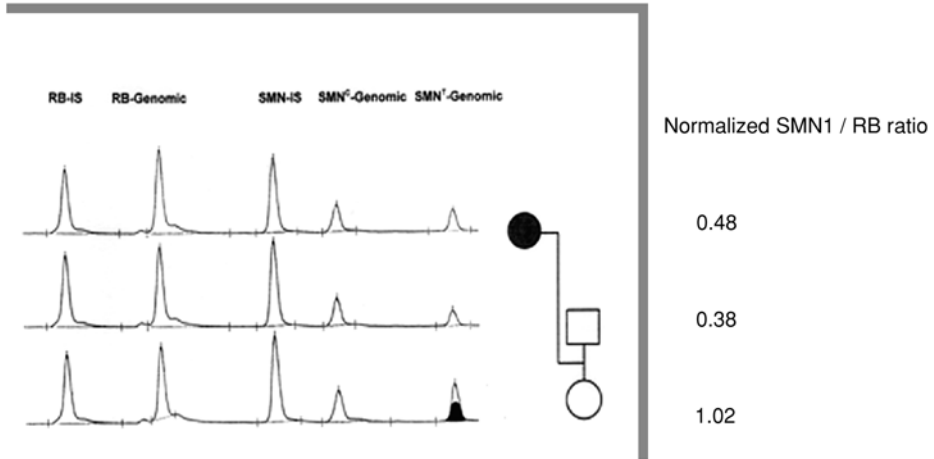


Fig. 2. Example of hemizygous SMN exon 7 deletion detection in an SMA family. The affected child is indicated by a black symbol. The origin of the signals is indicated on top. The normalized SMN1/RB ratio is indicated on the right. The normalized SMN1/RB ratios are indicated on the right. Note that ratios of approx 0.5 that are indicative of a hemizygous SMN exon 7 deletion have been detected in the affected child and the carrier father, and a ratio of approx 1.0 that are indicative of two SMN exons 7 have been detected in the mother.

Several quantitative assays based on competitive amplification of SMN1 and SMN2 exons 7 to determine their gene-copy number are now available (25–28).

### 1.2.2. De Novo SMN1 Rearrangements

SMN1 *de novo* deletions occur at a frequency of approx 2% of SMA patients, and the majority are of paternal origin (9,29). Testing of parents of homozygously deleted SMA patients may give additional information on *de novo* deletions. In the majority of cases, it has been shown that these *de novo* deletions are associated with a meiotic recombination or meiotic gene conversion. Thus, the recurrence risk in such families would theoretically not be elevated. However, Campbell et al. (30) reported on possible germinal mosaicism in SMA, which would lead to an elevated recurrence risk. In practice, these data should lead to prenatal diagnosis, despite evidence of a *de novo* mutation.

A complication/pitfall in the dosimetric analysis of SMN1 exon 7 is the occurrence of two (or more) SMN1 genes on a single chromosome 5 in about 4% of individuals, as observed by McAndrew et al. (25), Wirth et al. (31), and others. This affects the sensitivity of this technique for carrier identification, since

individuals with an SMN1 [1,1] genotype (a single SMN1 gene on each chromosome; non-carriers) cannot be distinguished from individuals with an SMN1 [0,2] genotype (both SMN1 genes on a single chromosome; carriers). Also, in carrier testing of individuals with no positive family history for SMA—e.g., testing of partners of known carriers—it should be taken into account that mutant non-deletion SMN1 alleles (approx 2.5% of mutant SMN1 alleles) will not be detected. The consequences for the sensitivity and specificity of carrier testing by a dosimetric method have been discussed (26,27).

Carrier testing in relatives of SMA patients—e.g., siblings and/or uncles and aunts—could also be performed by indirect analysis using flanking markers.

### 1.2.3. Indirect Carrier Detection by Linkage Analysis

Linkage analysis is useful for carrier testing if quantitative deletion analysis is not feasible. One prerequisite is the availability of sufficient DNA samples from family members for establishing linkage phase and informativity of the markers.

In postnatal applications, linkage-based testing of unaffected siblings, or preferably the grand parents of the index case, may be useful to identify a possible meiotic recombination associated with a *de novo* deletion or rearrangement involving SMN1. Also, uniparental isodisomy may be identified by the use of flanking markers.

## 1.3. Prenatal Testing

A third indication for DNA analysis is prenatal analysis. This is often requested by parents of a SMA patient, and may also be indicated for couples who have been shown to be carriers of a mutant SMN1 allele, or for couples of an affected patient with SMA with an identified carrier, which is not a rare situation in SMA type III.

Prenatal analysis is preferably carried out by direct mutation detection—e.g., by testing for a homozygous SMN1 exon 7 absence. One prerequisite is the identification of a homozygous SMN1 exon 7 absence in the index patient.

The homozygous absence of SMN1 exons 7 (and 8) in unaffected haplo-identical sibs of SMA patients have been described (13,14,32). The parents of a SMA patient may actually have a homozygous SMN1 exon 7 absence themselves. Although it is difficult to predict whether the parental mutant SMN1 allele that had not been transmitted to the affected child may have a pathogenic effect at all, it cannot be considered simply as a non-risk SMN1 allele. Such an allele should be treated as an allele with a potential pathogenic effect.

Prenatal testing based on linkage analysis is useful in families with a clinically ascertained patient with proximal SMA without a homozygous SMN1 deletion. This approach should only be used when a hemizygous SMN1 dele-

tion has been identified in the index case. Prenatal analysis in families with non-deleted patients should generally not be performed. Flanking markers may also be used to confirm the results of deletion analysis in prenatal testing, and to exclude an interfering admixture of maternal cells in the fetal sample.

## 2. Materials

Analytical-grade reagents should be used at all stages, unless otherwise stated.

### 2.1. Direct SMN1/2 Exon 7/8 Absence Test

1. 10X reaction buffer: 670 mM Tris-HCl, pH 8.3, 166 mM ammonium sulfate, 500 mM KCl, 37 mM MgCl<sub>2</sub>, and 0.85 mg/mL bovine serum albumin (BSA). Filter sterilize and store in 1-mL aliquots at -20°C.
2. Deoxynucleotide triphosphates (dNTPs): Dissolve 10 mg of individual nucleotides (Sigma, St. Louis, MO) in sterile distilled H<sub>2</sub>O to a concentration of 80 mM, and mix together to form an equimolar mix of all dNTPs. Store 500-μL aliquots at -20°C.
3. Oligonucleotide primers: The author uses commercially synthesized primers. These can be stored for several years at -70°C. Working solutions of 100 ng/μL should be prepared.
4. *Taq* polymerase. The author uses Perkin-Elmer (Foster City, CA) for this application.
5. Restriction enzymes. The author uses Amersham-Pharmacia (Uppsala, Sweden), but enzymes from other manufacturers may work equally well.
6. Use a mixture of ordinary electrophoresis-grade agarose (BCL, Boehringer Mannheim, Mannheim, Germany) and NuSieve low-gelling-temperature agarose (FMC, Rockland, ME).
7. Loading buffer: Per 100 mL: 15 g Ficoll 400, 250 mg bromophenol blue, 2 mL 0.5 M EDTA (pH 8.0) in sterile distilled H<sub>2</sub>O. Store at room temperature.
8. Agarose electrophoresis equipment: Pharmacia LKB-GNA100 or Pharmacia LKB-GNA200 (Amersham Pharmacia).

### 2.2. Dosage Analysis of Hemizygous SMN1 Deletions

1. PCR materials: Use the same materials described previously except for those described in the following steps.
2. Primer mixture RB1/SMN: a mixed working solution of 100 ng/μL for each primer should be prepared (*see Note 14*). Primer sequences are summarized in **Table 2**.
3. *Taq* polymerase: The author uses AmpliTaq (Perkin-Elmer) for this application.
4. Internal standard plasmids (*see also Note 7*): Prepare a mixture of the SMN-IS and the RB-IS according to **Note 15**.
5. Acrylamide: Use 19:1 polyacrylamide/*bis*-acrylamide mix. The author uses ALF-grade Ready Mix Gel (Amersham-Pharmacia).

**Table 2**  
**Sequences of Primers**

Test	Forward primer, 5'–3'	Reverse primer, 5'–3'
SMN1/2 exon 7 homozygous absence test (22) ( <i>DraI</i> will cleave SMN2 exon 7 specifically)	R111: AGACTATCAACTTAATTTCTGATCA	X7-Dra: CCTTCCTTCTTTTTGATTTTGTTT
SMN1/2 exon 8 homozygous absence test (22) ( <i>DdeI</i> will cleave SMN2 exon 8 specifically)	541C960: GTAATAACCAAATGCAATGTGAA	541C1120: CTACAACACCCTTCTCACAG
SMN1 exon 7 dosage analysis test (27)	R111: AGACTATCAACTTAATTTCTGATCA (fluorescently labeled)	X7-Dra: CCTTCCTTCTTTTTGATTTTGTTT
The RB1 gene reference product primers:	Rbex13F: ATTACACAGTATCCTCGACA	RBex13R: TATACGAACTGGAAAGATGC (fluorescently labeled)

For monitoring the efficiency of the PCR, cloned DNA of a SMN and an RB1 internal standard are added to the multiplex reaction (30). Clones containing internal standards are available from the author).

### 2.3. Linkage Analysis

1. PCR materials: Use the same materials as described in **Subheading 2.1**. Direct SMN1/2 exon 7/8 absence test.
2. Primers are described in **Table 3** (see **Note 13** for primer sequences).
3. Acrylamide: Use 19 : 1 polyacrylamide/*bis*-acrylamide mix. The author uses ALF-grade Ready Mix Gel (Amersham-Pharmacia).

There are also multi-copy markers, including C212 and C272 (Ag1-CA), which lie near the 5' end of SMN1 (**9,38,39**). These can also be used, although the interpretation of alleles requires considerable experience.

## 3. Methods

### 3.1. Direct SMN1/2 Exon 7/8 Absence Tests

#### 3.1.1. PCR Conditions

1. Prepare a reaction mixture of all components according to the following scheme into a sterile thin-walled 0.5-mL microcentrifuge tube (see **Notes 2–4**):

<u>exon 7</u>		<u>exon 8</u>	
10X reaction buffer	2.0 $\mu$ L		2.0 $\mu$ L
4 $\times$ 2 mM dNTP	2.0 $\mu$ L		2.0 $\mu$ L
100 ng/ $\mu$ L primer R111	1.0 $\mu$ L	100 ng/ $\mu$ L primer C960	1.0 $\mu$ L
100 ng/ $\mu$ L primer X7-Dra	1.0 $\mu$ L	100 ng/ $\mu$ L primer C1120	1.0 $\mu$ L
distilled water	12.5 $\mu$ L		12.5 $\mu$ L
1 U/ $\mu$ L <i>Taq</i> polymerase	1.0 $\mu$ L		1.0 $\mu$ L
500 ng/ $\mu$ L DNA	0.5 $\mu$ L		0.5 $\mu$ L

The different genomic DNA samples are added last.

Include a no-template control, and a positive (homozygous SMN1 exon 7 (and 8) absence) and negative (presence of SMN1 exon 7 (and 8); see also **Note 1**).

2. Place in a thermocycler with preheated block at 94°C.
3. PCR cycling conditions (32 cycles): 94°C for 1', 57°C for 1', 72°C for 1'.
4. Add 1  $\mu$ L 18 U/ $\mu$ L *DraI* to each exon 7 reaction, and add 1.5  $\mu$ L 8 U/ $\mu$ L *DdeI* to each exon 8 reaction.
5. Incubate at 37°C for 2 h.
6. Add 6  $\mu$ L loading buffer and mix.
7. Load on a 2% agarose gel (1% Nusieve/1% BCL agarose) containing ethidium bromide (5  $\mu$ g/mL).
8. Run at 150 V for 1 h.
9. When separation is complete, photograph the gel under UV transillumination.
10. Interpretation.

**Table 3**  
**Genetic Markers Flanking the *SMN1* Gene at 5q11.2–q13.3 (Note 13)**

Locus	Location	Recombination	PIC	Size
D5S679	Proximal	3%	0.63	180–222
D5S680	Proximal	3%	0.57	141–173
D5S125	Proximal	2%	0.38	143 and 147
D5S681	Proximal	2%	0.68	142–156
D5S435	Proximal	1%	0.68	128–144
D5S629	Proximal	1%	0.81	233–253
D5S823	Proximal	1%	0.54	128–150
D5S1556/D5F150	Intragenic/ <i>SMN1</i> promoter region	0%	0.98	90–122
D5S149	Intragenic/ <i>SMN1</i> promoter region	0%	0.99	166–203
D5S557	Distal	1%	0.46	148–172
D5S610	Distal	1%	0.80	106–124
D5S351	Distal	1%	0.74	196–234
5'-MAP1B	Distal	1%	0.76	100
3'-MAP1B	Distal	1%	0.72	212–226
D5S112	Distal	3%	0.74	94–120
D5S127	Distal	5%	0.84	96–114
D5S39	Distal	7%	0.74	212–220

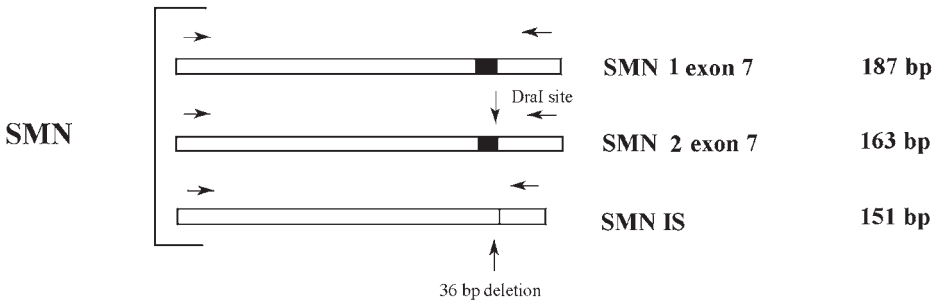
### 3.2. Dosage Analysis of Hemizygous *SMN1* Deletions (Notes 5-8)

A number of quantitative assays based on competitive amplification of *SMN1* and *SMN2* exons 7 to determine their gene-copy number have been described (25–28). The latter system is described in more detail here, and makes use of fluorescently labeled primers, enabling the analysis to be performed on an automated sequencer.

#### 3.2.1. PCR Conditions and Electrophoresis using an Automated Sequencer (Note 12)

1. Prepare a reaction mixture of all components according to the following scheme into a sterile thin-walled 0.5-mL microcentrifuge tube (a maximum of 39 samples can be run on a single gel):
  - 2.0  $\mu$ L 10X reaction buffer
  - 2.0  $\mu$ L dNTPs (4  $\times$  2.0 mM dNTP)
  - 1.0 primer mixture RB1/*SMN* (4  $\times$  100 ng/ $\mu$ L)
  - 3.0  $\mu$ L RB-IS/*SMN*-IS mix

## SMN test sequence



## RB reference sequence

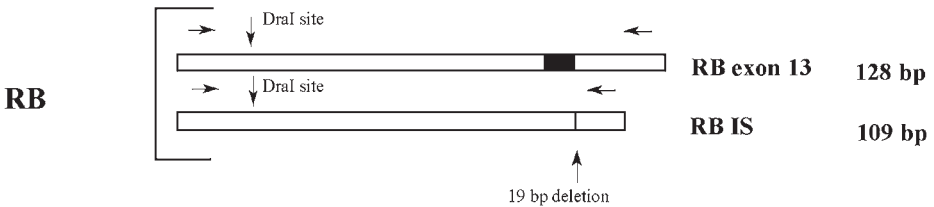


Fig. 3. Amplification strategy of the quantitative SMN exon 7 analysis. Deletion products of SMN exon 7 (36-bp deletion) and RB1 exon 13 (19-bp deletion) have been generated by PCR and subcloned into pCR2.1. These plasmids have been added as internal standards (SMN-IS and RB-IS, respectively). Three PCR-products are generated by the SMN primers: SMN1, SMN2, and SMN-IS. SMN1 and SMN2 can be separated by *DraI* digestion. Two PCR-products are generated by the RB1 primers: RB-genomic and RB-IS. The RB-genomic and the RB-IS contain a *DraI* restriction site, enabling the monitoring of the completeness of digestion. The forward SMN primer and the reverse RB primer are fluorescently end-labeled. The length of the PCR-products in bp is indicated.

- 1.0  $\mu\text{L}$  5X diluted *Taq* polymerase
  - 0.5  $\mu\text{L}$  25 mM  $\text{MgCl}_2$
  - 9.0  $\mu\text{L}$  distilled water
  - 1.5  $\mu\text{L}$  50 ng/ $\mu\text{L}$  genomic DNA
2. PCR cycling conditions: 94°C for 3', followed by 26 cycles of 94°C for 1', 57°C for 1', and 72°C for 1' (3' during the last cycle).
  3. Add 1.3  $\mu\text{L}$  *DraI* per sample, and incubate at 37°C ON.
  4. Dilute 3.0  $\mu\text{L}$  of each PCR product by adding 7.0  $\mu\text{L}$  distilled water and 10.0  $\mu\text{L}$  loading buffer containing a 200-bp internal standard (Amersham Pharmacia).
  5. PCR products are denatured for 3' at 95°C, and transferred onto ice.
  6. Load 5  $\mu\text{L}$  of the diluted PCR products on a 6% PAA gel using an ALF.



7. Electrophorese using the following conditions: voltage 2000 V; current 75 mA; power 50 W; laser power 2 mW; temperature 45°C, until the fragments have been separated sufficiently (approx 400 min; **Note 9**), buffer 0.6X TBE.
8. Calculate the peak areas using the Fragment Manager software (**Note 10**), and print the results.
9. Interpretation (**Note 11**).

### 3.3. Linkage Analysis (Note 13)

A number of informative microsatellite markers flanking the SMA gene are summarized in **Table 3 (31,34–37)**. It should be possible to find an informative proximal and distal marker in the majority of cases so that the overall error based on linkage with flanking markers is <1%.

#### 3.3.1. PCR Conditions

1. Prepare a reaction mixture of all components according to the following scheme into a sterile thin-walled 0.5-mL Eppendorf tube:
  - 3.0 µL 10X reaction buffer
  - 3.0 µL dNTPs (4 × 2.0 mM dNTP)
  - 1.0 µL 100 ng/primer
  - 21.5 µL distilled sterile water
  - 1 U/µL *Taq* polymerase
  - 0.5 µL 500 ng/µL genomic DNA
2. PCR cycling conditions: 94°C for 1', followed by 30 cycles of 94°C for 1', 55°C for 1', and 72°C for 1'.
3. Add to 1.0 µL of each PCR product 29 µL of distilled sterile water.
4. Mix 5.0 µL with an equal volume of loading buffer containing a 200-bp internal standard (Amersham Pharmacia).
5. Load the diluted PCR products on a 6% PAA gel using an ALF.
6. Electrophorese using the following conditions: Voltage: 2000 V; current: 75 mA; power: 50 W; sampling interval: 2 s; laser power: 2 mW; temperature: 45°C; runtime: 400 min, buffer 0.6X TBE.
7. Interpretation (*see Note 10*). Print the results.

### 3.4. SMN1 Point Mutation Analysis

SMN1 point mutation analysis is difficult because of the high homology between SMN1 and SMN2, and is beyond the scope of current routine DNA service for SMA. When analysis of point mutations is necessary, specialized laboratories that offer this facility should be contacted.

## 4. Notes

1. The gene nomenclature that has been accepted by the nomenclature committee is SMN1 for the telomeric (pathogenic) SMN gene and SMN2 for the centromeric (copy) gene. It is recommended that samples from patients known to have a homozygous SMN1 deletion (positive reference samples), as well as samples

- known to contain intact SMN1 allele(s) (negative reference samples), are always digested and run in parallel on gels to monitor completeness of the digestion, in addition to a no-template control reaction to exclude carry-over contamination.
2. Homozygous absence of exons 7 (and 8) of SMN1 can be detected by several alternative methods, including the originally described SSCP technique—either on polyacrylamide (8% on 12-cm gels works well) or MDE gels (10). The deletion can be visualized by silver staining of the gels as well as by radioactivity or fluorescence (33). SSCP offers the advantage that point mutations in the gene may be detected as well as deletions. Gene conversion can also be detected by SSCP analysis, depending on the local laboratory conditions used. The restriction digest method does not differentiate between a true deletion and a gene conversion, but correctly determines the absence of SMN1 exons 7 and 8 from their normal genomic position. However, the SSCP method will also identify neutral polymorphisms. In addition, a study from Wang et al. (32) has shown that 4% of asymptomatic family members carry a polymorphic variant of exon 7 of SMN1 (telomeric), which looks like a homozygous deletion on SSCP gels.
  3. Alternative restriction enzyme digestion (26): For exon 7, the enzyme *HinfI* could be used to separate SMN1 and SMN2 in combination with specific primers. The SMN1 exon 7 product will contain two *HinfI* sites; the SMN2 exon 7 product will contain one *HinfI* site. Both restriction-based tests are rapid assays, and offer the additional advantage that the pathogenic absence of SMN1 exon 7 (and 8) is very specifically tested for.
  4. Some labs prefer to confirm exon 7-only deletions by a second, independent test (in the majority of cases, the absence of both exons 7 and 8 is detected; these test results confirm each other). Testing for SMN1 exon 7 is the most accurate test, and some laboratories consider testing for SMN1 exon 8 to be redundant. Testing for SMN1 exon 8 only may lead to a false-negative result.
  5. Since in clinical DNA service practice the majority of the tests used are currently based on qualitative analyses, it is strongly recommended that implementation of a quantitative SMN1 assay should be preceded by a thorough validation of sensitivity and specificity by the laboratory that performs this testing.
  6. Reference samples with known SMN1 exon 7 copy number should be included on the PAA gels.
  7. The dosimetric SMN1 exon 7 assay makes use of internal standard plasmids that are added to the reaction mixture for monitoring of the efficiency of the amplification. For availability of these standards and internal controls, please contact the author of this chapter.
  8. New PCR technology—e.g., based on real-time PCR (such as Taqman or FRET technologies)—allows the quantification of the PCR during the course of the reaction. Applications for SMA are currently under development.
  9. Peak positions: RB-IS 109 bp, RB1 genomic (exon 13) 128 bp, SMN-IS 151 bp, SMN2 164 bp, SMN1 188 bp. The completeness of the *DraI* digestion can be monitored by the absence of peaks from undigested products of 237 bp (RB exon 13 genomic product) and 218 bp (RB-IS).

10. De ALF-Manager software operates under the OS/2 system. Data are stored in a file called x.ALF. The numbering is in chronological order starting with 1. This number must be entered by the operator. For gel number 300, the data (including running conditions) are stored in a file called 300.ALF. These ALF-files can be transferred to another workstation via a diskette, and analyzed using the Fragment Manager V1.2 software (Amersham Pharmacia).
11. Since there are two copies of RB per genome, the genomic SMN1/genomic RB ratio can be used to determine the relative copy number of SMN for all samples, and corrected for differences in amplification efficiency of the two competitive PCR reactions by multiplication using the factor RB-IS/SMN-IS. This corrected ratio is normalized for the mean of the ratios obtained in two standard reference samples (from individuals carrying two copies of SMN1 exon 7) that must be included in all gels. Also, two reference samples must be included in all gels from hemizygous SMN1 exon 7 carriers, as well as a sample lacking genomic DNA (no template control). Non-carriers have a normalized and corrected ratio over .75, and carriers have a normalized and corrected ratio under .75. This calculation takes into account a possible difference in amplification efficiency for the two competitive PCR reactions used. We have noted that reliable results are only obtained when the signals of the RB-IS, SMN-IS, and RB-genomic amplification products do not differ greatly. As a rule of thumb, the signal with the lowest intensity may not be less than 50% of the highest signal of the three PCR products.
12. All reactions must be carried out *in duplo*. Only those results in which the *duplo* reaction results do not differ more than 20% should be considered.
13. Primer sequences can be found in the Genome Database (<http://gdbwww.gdb.org>).
14. It is advisable to prepare small batches of primer mix working solution, and test in advance whether equal amplification results for RB and SMN are obtained, prior to preparing a larger working solution.
15. Theoretically, the mixture of SMN-IS and RB-IS should contain equimolar amounts of plasmids, and 15,000 molecules of each plasmid should be added to the reaction mixture. However, since their amplification efficiencies may differ because of differences in purity, it is small amounts of different ratio mixtures should be prepared using a X: 1.0, 1.25:1, 0.25:1, 0.5:1, 1:1, 1:0.5, 1:0.25, 1:0.125, 1:X dilution series starting from approx 100 pg/ $\mu$ L of each plasmid, and perform series of test amplifications adding 3  $\mu$ L of each dilution of a 1:1 dilution series of these different ratio mixtures. When adequate results are obtained—e.g., when approximately equal genomic RB, genomic SMN1/2, RB-IS, and SMN-IS amplification signals are seen—a large batch of mixture should be prepared with an approximate concentration of 5,000 molecules/ $\mu$ L of each plasmid and stored in 150- $\mu$ L aliquots at  $-20^{\circ}\text{C}$ . Per reaction, 3  $\mu$ L of this mixture should then be added.

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## Quality Management in Molecular Genetics

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### 1. Introduction

#### 1.1. History

In the last decade, the demand for molecular genetic testing has increased enormously. Many of the laboratories that offer diagnostic molecular genetic testing on a routine basis originated from a research-based setting. The exponential growth of the clinical diagnostic molecular genetic laboratories (CDMGLs), together with improving technical approaches, has forced these laboratories to reorganize and standardize their methods and procedures, and to manage their increasing dataflow more efficiently. Better management of dataflow is important for confirmation of a clinical diagnosis. In most cases, direct mutation analysis of patients DNA now allows a definitive determination of carrier status or the risk of a fetus being affected. The results of DNA diagnostic tests are thus of major importance in clinical decision-making. Therefore, the quality of the whole laboratory process—from sample reception to reporting of the results, calibration of equipment to training of personnel, and documentation to method validation—should be managed systematically.

This chapter provides an introduction to various aspects of quality management in a CDMGL, highlighting some of the essential elements and benefits of a good quality system, and offering some examples of managing quality in daily practice.

#### 1.2. Deming Circle

The American statistician W. Edwards Deming (*I*) developed a model for integrated quality care. Deming visualized a circle showing the constant process



of quality care divided into four important phases, with statistical methods to analyze the third phase:

Phase 1: PLAN

Phase 2: DO

Phase 3: CHECK

Phase 4: ACT

Following the act phase, Deming suggested that it is important to develop new plans and then once again enter the first phase of the circle before proceeding to all the following phases. Using this model, quality management is a process of continual improvement.

Several other approaches have been developed to bring about the practical realization of the four phases of the Deming circle. Best-known are those by Crosby (2), Juran (3), Feigenbaum (4), and Masaaki Imai (5). Crosby (2) developed the plan-phase, Juran (3) concentrated on the do-phase, and Feigenbaum (4) specialized in the act-phase. Masaaki Imai (5) describes the optimal use of the intellectual capacity of all the people in an organization. Together, these authors elevated the concept of quality from the primary process of producing high-quality goods to a higher level in an organization, including its management.

### **1.3. Organization**

A strong commitment to raising standards is needed by laboratory management for the introduction of quality management in an organization. Developing a good-quality policy is the responsibility of management, but the attainment of quality requires the participation of all the members of an organization. The International Standards Organisation's (ISO) standard ISO 9000 (6) defines quality management as "that aspect of the overall management function that determines and implements the quality policy." The definition of this standard includes the following two notes:

1. "The attainment of desired quality requires the commitment and participation of all members of the organization whereas the responsibility for quality management belongs to top management."
2. "Quality management includes strategic planning, allocation of resources and other systematic activities for quality, such as quality planning, operations and evaluations."

This definition shows that the organization of a laboratory and the responsibilities of the different members within that organization are important for quality management.

The organization that implements a quality management process can be held legally responsible, and it is therefore important to chart an outline of the hierarchic and organizational structure of the laboratory. If the formal and infor-



mal functional and communication lines differ from the hierarchic lines, it is useful to draw these in a separate chart. The managerial and technical personnel of the organization should have the authority and resources needed to carry out their duties correctly. Job descriptions should be updated regularly to clarify and reinforce the staff's duties and responsibilities within the laboratory, and documented training procedures should be in place. A CDMGL produces a large volume of test data, and the scientist or technician who performs the test is often the first person to interpret the results. It is common practice in many laboratories that the results and their interpretation are authorized by a second suitably qualified person, independent from the scientist or technician performing the test. A fully interpretative report on the results is usually forwarded to the referring clinician by the scientific staff.

The workload of CDMGLs is gradually increasing in line with new advances in technologies and research (mutation analysis is often extended from a hot spot to the whole gene, and for some clinical indications more genes must be analyzed completely). Thus, there is a continual need for additional extra well-trained personnel. Laboratory management must also ensure that there are clear criteria defining who is able to perform the second independent check of data. To ensure that the quality management system is implemented and adhered to, laboratory management should appoint a laboratory quality manager with a defined role and responsibilities and the authority to implement them. The quality manager should be independent, but should report to laboratory management and participate in the laboratory policy and resource decision-making process. It is advisable for the laboratory management to appoint deputies for key managerial personnel such as the quality manager and head of the laboratory.

#### **1.4. Standards**

Standards are an important part of quality management. The process that enables a laboratory to gain recognition from an external agency that its activities and products are of guaranteed high quality and meet the set standards is called accreditation. Accreditation involves an external audit of the ability of the laboratory to provide a service of high quality. By declaring a defined standard of practice and having this independently confirmed, accredited laboratories attain a hallmark of performance and offer reassurance to users of their service. CDMGLs can choose to comply with various national or international standards that can be accredited by different independent national accreditation bodies such as the United Kingdom Accreditation Service (UKAS), Clinical Pathology Accreditation (UK) Ltd (CPA), RvA, or The Dutch Accreditation Board for Medical Laboratories (CCKL) in the Netherlands. There is a growing trend toward complying with the international standards ISO: 17025 (7) for testing and calibration laboratories and a new standard 15189 (8) for medical laboratories.

Both of these place increased emphasis on the laboratory output, the genetic test report in the case of the CDMGL, and being “fit for purpose” and responsive to the needs of the user. These standards also place a greater emphasis on managerial and technical requirements, and require laboratory management to establish a defined Quality Policy committing it to continual improvement and user satisfaction. This Quality Management System must be documented in a Quality Manual that outlines the quality policy and the laboratory’s procedures and practices. There must also be mechanisms for Quality Audit, Quality improvement, and Document Control. The Quality Manager should implement this process and help the laboratory to maintain compliance with the quality standards.

## 2. Quality Controls

### 2.1. Definitions

A common quality policy implemented by many laboratories is the use of internal quality controls to ensure a high standard of test results, and the subsequent release of valid and reliable test reports. In general, there are three types of quality controls, and use of these controls will further enhance the standards of the validated test result:

1. **First-line controls:** A constant check of the results of the tests performed by the technician. These controls are a check on the repeatability of test results.
2. **Second-line controls:** A check on the spread of the results produced within a laboratory, and therefore a check on the reproducibility of test results.
3. **Third-line controls:** A check of the spread of the results produced by different laboratories.

In general, a method is qualified as valid if a minimum of two line controls of the three possible controls are defined and performed on a regular basis. Two further parameters should also be considered when validating the reliability of the test result:

1. **Repeatability:** A measure of the spread of the results, produced with the same method on identical sample materials under *the same* circumstances (e.g., same technician, same equipment, same laboratory, at approximately the same time).
2. **Reproducibility:** A measure of the spread of the results, produced with the same method on identical sample materials under *different* circumstances (e.g., different technicians, different equipment, at different times).

### 2.2. Daily Practice

The different quality controls defined previously should be phased in and translated into the daily practice of a CDMGL. Examples of quality controls in daily use include:

### *2.2.1. First-Line Controls*

PCR blanks (no DNA template added to the PCR reaction) to check for contaminations, size markers to check for correct length of the PCR products or DNA digests, visual checks (e.g., clear lysate in DNA isolation, equal reaction volumes in 96-well plates), and normal controls (DNA samples with no mutation in the genes being tested).

### *2.2.2. Second-Line Controls*

Analysis of two duplicate DNA extracts from the same blood sample isolated in two separate rounds (this is a check of the reproducibility of the DNA isolation method and on sample swaps), repeated testing of positive control samples (DNA samples with a known mutation in the gene being tested), correct segregation of haplotypes in a family, the use of validated immortal sources of DNA such as lymphoblastoid cell lines, and the use of CEPH 134702 as a reference sample for haplotyping.

### *2.2.3. Third-Line Controls*

Laboratories can check the performance and spread of their results, quality controls, and procedures by participating in organized external quality assessment (EQA) schemes for molecular genetics. Examples of such schemes include those organized by the European Molecular Genetics Quality Network (EMQN), the United Kingdom National External Quality Assessment Scheme (UKNEQAS) for molecular genetics, and the European Cystic Fibrosis Thematic Network (ECFTN). The performance of the laboratory is evaluated on the basis of its ability to correctly genotype DNA samples in the context of a mock clinical question, and to provide a full interpretation of the test results. There is a move toward EQA schemes also evaluating the clerical accuracy of reports—a practice that has been running in the UKNEQAS scheme since 1997. Evaluation of the results of EQA schemes is often guided by Best Practice Guidelines (9) that are a consensus statement by the diagnostic molecular genetics community on the best approaches to take in the molecular diagnosis of a disease.

The use of first- and second-line controls may vary depending on the reason of referral. For example, mutation analysis in a family with a known mutation should always include a positive and a normal control. The approach used to detect the mutation should be a direct technique such as sequence analysis, Southern blotting, or mutation-specific PCR. However, several choices are available for requests for mutation analysis in a family with a still undetermined mutation, so-called mutation-scanning analysis. Whole-gene scans should be done in large batches with many samples. For gene scans using direct sequenc-

ing in genes without mutational hot spots, a minimum of two samples in a scanning series will suffice to obtain a normal pattern comparable to a variant pattern. Thus, there is no need for a separate normal control sample that reduces the costs of testing.

In order to demonstrate the efficacy of the test, when possible, laboratories should include at least one positive control for every region scanned. Positive controls are of major importance when using *indirect* mutation scanning techniques such as denaturing gradient gel electrophoresis (DGGE) or the protein truncation test (PTT) and in the validation of the methods prior to their implementation in routine diagnostic procedures. The positive controls used in most laboratories consist of collections of DNA samples from patients with previously identified mutations, and many of these samples have been immortalized as lymphoblasoid cell lines and represent an important resource for the laboratory. However, laboratories cannot be expected to have positive control samples for every region scanned! To address the need for more positive controls, a multi-center project funded by the European Commission was initiated in 2001 to prepare and validate certified reference materials (CRMs) for clinical molecular diagnosis. The CRMGEN project will develop certified reference materials using immortalized cell lines from different patients with well-characterized different mutations, cloned DNA-fragments, and artificial DNA fragments.

All laboratories that use quality controls should be aware of the European Union's directive on the use of In Vitro Diagnostic Devices (IVDs) (*II*). The IVD directive makes it illegal to distribute control samples between laboratories and in diagnostic tests unless they have been certified as reference materials.

### **3. Quality System**

#### **3.1. Benefits**

There are many reasons why a laboratory may decide to implement a quality management system and seek accreditation for the service they are providing. For many private-sector laboratories, the benefits are predominantly commercial. An accredited laboratory is at a competitive advantage against a laboratory that is not accredited, and therefore more likely to acquire a greater number of clients and testing requests. However, in public-sector laboratories, the main benefits are not commercial, but instead tend to affect the laboratory and its users. So, in the case CDMGLs, there are two main user groups: the patients and the referring clinicians. The referring clinicians are an important part of the diagnostic process. A laboratory has a duty to the patient and the clinicians to provide them with a service of high quality. This affects all aspects of the process, from blood sampling to communicating the results of the final lab report to the patient.

The implementation of a quality management system in a laboratory involves a period of major disruption, and can have a significant impact on the job of the referring clinicians. For example, the criteria for accepting a blood sample are often narrowed, and laboratories tend to adhere more consistently to these criteria. The implementation of a quality system is often the beginning of a stricter, more formal sample acceptance policy. The referring clinician will be asked to fill out the forms completely and to label sample tubes correctly. The best practice guidelines for laboratory internal quality control (**10**) produced by the EMQN suggest that the sample tubes accompanying a referral should have at least two pieces of unique information to link them together, such as a barcode, name, date of birth, and national insurance or hospital number. Incomplete forms and incorrectly labeled tubes often make a sample unacceptable for testing.

The formal acceptance of a quality management system can have significant rewards. Referring clinicians and patients can trust that all tests are performed using “state of the art” methods, complying to international standards and safeguarded as much as possible against sources of error such as sample swaps and technical mistakes. The reporting process is much improved, with less ambiguity and clearly defined turn-around times. There are also other benefits, including reduced error rates, clear privacy regulations, smaller audit trails, standardization of the diagnostic process, and external quality assessment of methods. However, the overall reward is the improvement to the service that the laboratory offers to its users.

The primary factor that motivates a laboratory to implement a quality management system is the desire to perform better. Implementation of quality controls and thorough validation of all diagnostic procedures leads to a reduction in errors and repeated testing because of failed or bad test results. Furthermore, a quality management system forces a laboratory to write down its Standard Operating Procedures (SOPs) to facilitate better and more efficient training of all staff. All SOP's should to be revised on a regular basis, making the organization less vulnerable to changes in staff and keeping technical know-how within the organization. Good management of the supplies process (such as ordering of buffers, primers, kits, and enzymes) will prevent delays in the diagnostic process, and should include the registration of batch numbers because this helps to solve technical problems. All laboratory equipment should be regularly calibrated to prevent a gradual decrease in quality and the potential failure of test results. Standardized filing and back-up of all data and technical records guarantees an audit trail and the possibility of repeated testing under nearly the same conditions. Finally, the job descriptions of all personnel involved in the laboratory should be reviewed annually and updated when necessary. All these factors ensure the efficient and responsible management of the CDMGL.

### **3.2. Document Control**

All documents issued to personnel in the laboratory as part of the quality management system should be reviewed and approved for use by authorized personnel prior to their issue. There should be an overview of the current revision status and distribution of documents in the quality system to preclude the use of invalid documents. Authorized editions of appropriate documents should be available at all appropriate locations. All documents should be reviewed periodically and revised if necessary. In general a review frequency of once a year is recommended. The regular review of documents should be seen as the shared responsibility between all members of staff and is best organized by distributing documents to different members of staff after first considering their daily tasks and experience with the procedures or methods described.

The different quality management standards require that identifying information about each document be displayed boldly, including the title, date of issue, revision date or number, total number of pages, authorization name and signature, and a unique file name or other identification if the document is being stored electronically on a computer database.

Changes to all documents should be reviewed and approved by authorized personnel, and all revised or new text is identified in the document. Obsolete documents should be removed from circulation, and a memo should be sent to all staff to notify them of this action. A copy of all controlled documents should be archived for later reference.

### **3.3. Internal Audits**

Audit is an important part of any quality management system. International standards require that laboratories periodically conduct internal audits of their activities to verify that their operations continue to comply with the quality system. An internal audit program addresses all elements of the quality management system. It is normal that all the main elements of the system are subject to internal audit once every year. All audit findings, including non-compliances, are recorded, and corrective actions are outlined. It is the responsibility of the quality manager to plan and organize the internal audit program. However, management is responsible for the implementation and followup of any necessary corrective actions. Suitably qualified staff should carry out audits, and if possible, staff should not audit their own activities.

There are several approaches that management can take to encourage staff participation in audit. One approach is to train a group of staff as internal auditors and make internal audit a formal event. The advantage of this approach is that any identified non-compliances are taken seriously, monitoring the labo-

ratory to take corrective action. The disadvantage is that it isolates a number of key staff members and potentially increases their workload significantly. Another approach is to let everybody in the laboratory participate in the internal auditing process and make it an informal, routine part of laboratory life. The advantage of this approach is that all staff learn about all the different activities and procedures in the laboratory, stimulating their commitment and enlarging the problem-solving capacity of the entire laboratory. The disadvantage is that it can be difficult to manage this approach.

Setting up a quality management system takes time. On average, it takes 1 yr to set up for a small-sized laboratory with less than ten employees, and up to 3 yr for a CDMGL with about 25 employees. The time needed to implement a system depends on many different factors. A major component of the successful implementation is the appointment of a suitably qualified and dynamic individual to the role of quality manager. The starting point for the laboratory is the writing of the SOPs that describe all the standard laboratory methods and procedures. This task is best shared between all staff members. Depending on experience, it is advisable to split the technical SOPs into basic technical procedures describing the methods used (e.g., DNA-isolation, sequencing, DGGE, setting up a PCR or PTT) and disease-specific protocols, describing the total procedure and referring to these basic methods. Other members of the organization can start with the introduction and maintenance of records for each item of laboratory equipment that contributes to the performance of the tests and the organization of preventive maintenance and calibration of critical equipment. One role of the quality manager is assisting the laboratory management in the development of a quality policy, discussing non-compliances to the standard(s), and the writing of a quality manual. An internal audit program should proceed once the bulk of SOPs are published. These first audits help to improve the SOPs and to change the laboratory culture from a research setting to a more professional diagnostic setup in the laboratory, preparing it for the process of inspection and gaining accreditation.

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## Regulation of Genetic Testing in Clinical Practice

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### 1. Introduction

Genetic testing is often regarded as a laboratory procedure in a molecular genetic laboratory. However, for practical use in health care, this definition is too narrow. In the majority of cases, the clinician is not using a genetic test for diagnostic purposes, but confirming or excluding a clinical suspicion of an underlying disorder of presumed genetic background. As an example, a confirmatory test could be requested by a pediatrician, who—after investigating an unhealthy child physically and with non-genetic tests—has the suspicion that the child has cystic fibrosis (CF) and orders a cystic fibrosis transmembrane conductance regulator (CFTR) mutation-scan. If mutations are found, the diagnosis is confirmed. Furthermore, the specific mutations detected may give prognostic information about the child's future health. The doctor will also have information that can be used for family investigations, and possibly for prenatal diagnosis. However, if only one or no mutations are found, this does not exclude the possibility that the child has CF. The test used may not detect all mutations, or the gene may have been silenced by other reasons—e.g., altering the regulation of the gene. An example of exclusion testing is a patient presenting with neurological symptoms, leading to a suspicion of Huntington's disease. A genetic test revealing a normal set of trinucleotide repeats in the IT-15 gene will indicate that the symptoms are not caused by this disorder. In genetic testing, the information given to the patients and their families must be an integral part of the process.

Therefore, in clinical practice genetic testing must be seen in a wider context and may be defined as follows:

“Genetic testing is the process of information transfer within which the laboratory investigation is integral. It consists of any analysis of family data, of human chromosomes, DNA, RNA, protein or certain metabolites to detect heritable or acquired disease and related genotypes, mutations, phenotypes, or karyotypes for clinical purposes. Such purposes include diagnostic, predictive, carrier, prenatal, preimplantation, or new-born testing, thus predicting risk of disease, identification of carriers establishing, prenatal or clinical diagnoses or prognoses in individuals, families or populations” (1).

Such a wide definition encompasses all aspects of the testing procedure, including genetic counseling both before and after a genetic test is performed.

The goal of this chapter is to highlight some of the issues connected with the regulation and the use of genetic testing in clinical practice.

## **2. Regulations/Guidelines/Statements**

Laboratory genetic testing of suspected constitutional genetic disorders is legally more regulated than other medical investigations, in order to protect family members from discrimination or misuse, mainly with regard to presymptomatic diagnosis.

### **2.1. International Documents**

Regulation of genetic services differs between different countries. In some, strong national regulations exist, whereas many countries have weak or a complete absence of regulations. However, a number of international bodies have published recommendations about individual rights as they relate to the development of genetic service. Important examples are the “Convention for the Protection of Human Rights and Dignity of the Human Being with Regard to the Application of Biology and Medicine” by the Council of Europe (2), the “Universal Declaration of the Human Genome and Human Rights” by UNESCO (3), and the “Proposed Guidelines on Ethical Issues in Medical Genetics and the Provision of Genetic Service” by WHO (4). Other examples are the HUGO “Statement on Principled Conduct of Genetics Research” (5), the “European Society of Human Genetics’ Statements by the Public and Professional Policy Committee” (6), the Guidelines for quality assessment in prenatal diagnosis by the EU-concerted action EUCROMIC (7), and the documents from the recent workshop on “Genetic Testing in the New Millennium” organized by the OECD (Organization for Economic Cooperation and Development) (8,9).

### **2.2. National Documents**

An overview on existing regulations in Europe and the United States was made by the European Society of Human Genetics’ Public and Professional

Policy Committee in a background paper to a workshop on Provision of Genetic Services in Europe (Helsinki, September 2000) (6).

For those who work in countries with no or only rudimentary regulations who need to keep up with high international standards, these policy documents provide valuable information. A genetic testing laboratory operating in a relatively unregulated environment may benefit from having a written policy and guidelines based on these international documents and making their policies available to their clients. For example, the U.S. Clinical Laboratory Improvement regulations are available on the web (10) and constitute a sound basis for the provision of service.

### **3. Ethical Issues in the Molecular Genetics Laboratory**

There are no specific ethics for molecular genetics or molecular genetic tests, or for molecular genetics laboratories. The same premises and guidelines for good laboratory practice current in other medical laboratories should be achieved. However, in performing genetic investigations, many of the ethical issues become more evident. In addition to providing information that is highly predictive for future disease, molecular genetics tests often give information that is relevant to the person tested as well as to their relatives.

#### **3.1. Informed Consent**

Informed consent is of primary importance in in presymptomatic testing and in other uses of genetic testing. The result of the genetic test may be of importance, not only to those tested but also to their relatives and the establishment of a genotype is usually a “once-in-a-lifetime-test.” For these reasons, the test provider must ensure that the patient or client has full information on the consequences of the test results. For diagnostic tests, the clinician should always inform the patient, or if applicable, the parents, children, or spouse, about the clinical suspicion of a heritable disorder. It is the obligation of the clinician who is responsible for referring the test sample to the laboratory to have an informed consent from the patient when presymptomatic testing is requested (11). The laboratory may request a clear statement from the referring clinician with the referral that the patient has given their informed consent for the test.

#### **3.2. Ethical Committees**

In research laboratories, testing is most often performed as a part of the research program, and thus, ethical committees monitor the investigations. In clinical work, this is not mandatory. However, in a clinical laboratory, the staff may need to consider ethical implications. For example, if non-paternity is detected unexpectedly, or when analyzing the outcome of a newly introduced

diagnostic test. Therefore, any laboratory is advised to have an ethical advisory board, or at least be able to consult ethically trained persons.

#### **4. Purpose of Testing**

A genetic test may be diagnostic, presymptomatic, prenatal, or carried out for screening purposes. These scenarios require different approaches, and the purpose of the testing must be made clear for the laboratory by the referring clinician.

Urgent tests such as prenatal and pre-symptomatic tests should have as short a turn-around time as possible. The mutation to be tested should preferably be known in advance, and the sample should be tested together with a sample from a relative with that particular mutation as a positive control.

The target turn-around time should be clear to the referring clinicians, and should be related to the realistic and normal capacity of the laboratory. For some complex molecular investigations such as for colon cancer or breast cancer, the necessary time period may exceed several months, whereas for Huntington's disease, a normal turn-around time can generally be measured in weeks.

For more rare disorders, especially when service is offered as a part of a research program, the time to results may be extended. In these cases, the referring clinician and the patient or family involved must be fully informed about the progress of testing to avoid giving false hope to the patient and harming future contacts.

Screening tests should have a high predictive value—the absolute majority of those at risk should be detected with as low a number of false-positives as possible. The screening test should also be cost-effective, and only performed if there is a treatment available for the disorder tested. Moreover, clinical resources for proper follow-up should be available. The methodology used should be targeted for large-scale analysis, and a positive result should always be confirmed by a diagnostic test. Screening tests could be neonatal, prenatal, or performed later in life. The screened group could be a whole population, or a subset selected on the basis of calculated risks. The decision to start such programs must carefully consider the costs and benefits, and should be made as a part of a public health care system. More information on the prerequisites for genetic screening is available through the statements of the European Society of Human Genetics (6).

#### **5. The Importance of Genetic Counseling**

A genetic test is always related to clinical findings in a patient or to a person's family history. Based on this information, the referring clinician decides what type of test should be performed. Genetic counseling should be offered both before and after a genetic test is performed, and this is equally important

whether a mutation is detected or not. One of the common misunderstandings is that when a genetic test does not reveal a mutation, the gene cannot be involved as a cause of the disorder—an interpretation that may be deleterious to the patient (*II*).

Depending on the indication for investigation, pre- and post-test counseling is of varying importance. Whereas in the case of a diagnostic test for CF the post-test counseling to the patient and/or the parents may be the most important factor, in the case of a presymptomatic test for Huntington's disease, the pre-test counseling is of equal importance. In this situation, the clinician is dependent on the accuracy of the laboratory report. It is important that the results are clearly stated and interpreted in a way that is understandable for the non-specialist to ensure a high standard of genetic counseling.

## **6. Training and Workload of Laboratory Staff**

Effective staffing is a prerequisite for providing high-quality service. This includes both appropriate training and qualification for the personnel who perform the analyses and supervision, as well as a level of staffing that enables the laboratory to present results without unnecessary delay. If not a part of the laboratory staff, medical expertise must be available on a regular basis. The laboratory should also have policies for referral elsewhere in cases that require specialized expertise that it cannot provide.

Staff should be offered continuous education on a regular basis that is relevant to their position in the laboratory. Their competence should be documented and regularly updated.

The workload of each staff member is difficult to estimate, as it is influenced by many factors such as the degree of automation and the type of samples processed in the laboratory. However, the number of staff should be sufficient to ensure that no unnecessary delay occurs in the processing of the samples. Sufficient staff to ensure continuity of service in a clinical laboratory during absences and vacations is recommended, and lack of space or appropriate equipment must not be a quality-limiting factor.

## **7. Validation of the Laboratory—Quality Issues**

The validation of the laboratory should focus not only on process, but also include staffing, education of staff and supervisors, reporting, and recording. It should include external and internal quality assessment, with emphasis on standardization of operational procedures using recognized laboratory standards. The EMQN best practice protocols are very useful (*12*), as are the chapters of this book.

To maintain public confidence to reach high standards and to avoid abuse of molecular genetic investigations, participation in External Quality Assessment

activities, including a transparent organization and a willingness to allow public inspections of the laboratory, are necessary.

## **8. Clinical Significance of Test Results**

The clinician who sends samples to the laboratory may have little knowledge about how the work is performed in the laboratory. The results must always be written with this in mind so that the interpretation of the laboratory results is absolutely clear. Reporting of polymorphisms may sometimes cause problems. It may be difficult for the clinician to understand the nature of the molecular finding, and the clinical significance of the absence of a mutation. An even greater problem is when a finding—for example, a missense mutation—not previously described and of unknown function is reported. The clinician must have full information, especially if the laboratory requests an extended family investigation to be able to further delineate the clinical significance of the molecular findings. In these cases, careful, repeated genetic counseling is often required to avoid the risk that the patient and their family will misunderstand the clinical significance of the molecular findings.

## **9. Transition of Research to Clinical Testing**

Many research laboratories offer genetic testing to families who have contributed to their work, and to other families or clinicians asking for help with molecular diagnosis. Although the core members of the research families often are well-informed about the disease and risks and benefits of genetic testing, this may not be the case in the extended family. In addition, many researchers and scientists may not be trained to talk to patients and family members about presymptomatic or prenatal testing. Genetic testing in a research context should include a clinical geneticist or genetic counselor in the team to communicate information to family members.

As research progresses, the research scientist must make sure that a clinical laboratory can continue to offer a service to patients and families. The researcher, in collaboration with the health care providers, must develop a mechanism that allows the transition from research to clinical service.

For the families and patient organizations who have been involved in research, it is traumatic to realize that tests that have been offered suddenly are not available. A failure in this transition of research into clinical service may detract from families' interest to participate in future research.

Several factors must be considered in the decision to transfer a genetic test from research to clinical practice: Is there a need for a clinical test? Are the methods used reliable, and how will the test be validated? There is a danger of offering inadequately evaluated, unregulated tests of potential, but unproven value as a service. The researcher has the responsibility to have an open

discussion about the benefits and drawbacks of the clinical practice of his/her research.

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