High-Throughput Screening for the Detection of Unknown Mutations: Improved Productivity Using Heteroduplex Analysis


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The detection of specific disease-causing mutations in DNA is an important adjunct to successful genetic counseling in families with a history of genetic disorders and for prenatal diagnosis. Researchers performing routine mutation detection on large multi-exonic disease genes face a huge and laborious task, especially in the absence of regional clustering of mutations within the gene. Neurofibromatosis Type 1 (NF1) affects more than 20,000 people in Britain alone, however, the causative mutations in the vast majority are unknown. The NF1 gene comprises 60 exons and covers a genomic region of 350 kb (10). Previous studies indicate that screening all 60 exons might be necessary to ascertain more about the type of mutations present and the possible genetic mechanisms involved in causing the condition. The labor-intensive nature of many current molecular biological techniques is all too apparent, and although dramatic advances in automation have recently been achieved, their cost makes them inaccessible to many researchers. Since a large number of samples must be analyzed quickly and economically, an accurate, high-throughput mutation detection method is essential.

A well-designed robust assay should provide the basis for a large screening program, and some consideration was given to the various options available.

Reviews (4,5) indicate that unfortunately, many of the most sensitive mutation detection techniques (95%–99% detection rate) are also the most labor-intensive, with low sample throughput, and are usually complex and costly. In addition, they often require purification, restriction enzyme digestion, involve the use of radiochemicals or toxic chemicals and utilize expensive specialized equipment. Single-stranded conformation polymorphism (SSCP) (12) and heteroduplex analysis (HA), first described by Nagamine et al. (11), although not being the most sensitive methods (75%–90% mutation detection), have few of the above disadvantages. The major limitation of these two techniques, however, is the small size range of the polymerase chain reaction (PCR) products that give maximum sensitivity; 100–300 bp for SSCP (6) and 100–400 bp for HA (2), with loss of sensitivity outside these ranges. Thus, HA would appear to be the most suitable method for large-scale screening for mutations in the NF1 gene in which the size range of the exons give PCR products from 190–549 bp. This method has been used to detect 9 novel mutations in a 549-bp fragment (10) and many others identified in smaller PCR fragments (1,16,17). We investigated whether sample throughput could be greatly increased without adversely affecting the sensitivity of the HA technique.

MDES™ gel (AT Biochem, Malvern, PA, USA) is a unique polyacrylamide-based matrix that has been shown to give improved resolution and reproducibility in the detection of both SSCP and heteroduplex bands (3,15). Gels are subsequently visualized by silver staining, which is a more sensitive method than ethidium bromide staining (9). The most time-consuming component of this method is the silver-staining step, thus if the number of samples analyzed per gel could be dramatically increased, fewer gels would be required, therefore greatly increasing cost effectiveness. Previous attempts to improve sample throughput have involved the pooling of DNA samples (8) or the performance of multiplex PCRs (13).

Optimal resolution on MDE gels is obtained by electrophoresing DNA at least 30 cm through a 40-cm-long gel. Two PCR products were combined in each pool; any more may compromise sensitivity. Heteroduplex bands can be missed when analyzed on a 0.4-mm-thick gel because of their reduced intensity compared to the homoduplex band (2); therefore, samples are run on a thicker gel. Using a 32-tooth, well-forming comb, pooled samples were run in 30 wells (60 separate PCRs), with the two outer wells reserved, one for a 1-kb DNA ladder (Life Technologies, Paisley, Scotland, UK) and the other for a positive control sample (known mutation). Instead of performing complex multiplex PCRs, which is possible for HA, we investigated the use of sequential loadings. Heteroduplexes usually migrate slower than the corresponding homoduplexes, forming mutant bands less than 1 cm away, and...
this should be compatible with subsequent loadings. Although HA has been reported to be unsuitable for use in high-throughput, low-cost procedures (14), we find that the pooling of samples and performing five sequential loadings on each gel reduces the number of gels required by a factor of ten.

Different exons can be analyzed on the same gel provided that they either have similar migration characteristics on MDE gels or that they are not loaded in the same wells. Two adjacent exons can be screened simultaneously if separated by a small intron, thus ensuring that the final PCR product is within the optimal size range for screening by this method.

This novel use of multiple loadings is currently being used to systematically screen over 480 NF1 patients for the presence of mutations localized across the NF1 gene.

Each PCR was performed separately, as described previously (16). To ensure equimolar amounts of each PCR product when pooling samples, 2 µL of each product were electrophoresed on a 2% agarose gel (Boehringer Mannheim, Lewes, England, UK) containing 4 µg/mL ethidium bromide, and the DNA concentration was estimated visually. To prepare pools of PCR products for the first loading, approximately 150 ng each of two PCRs were added to 0.6 µL of 0.1 M EDTA (Sigma Chemical, St. Louis, MO, USA) to inactivate any residual DNA polymerase (2). Samples were denatured at 94°C for 3 min then transferred to a heating block at 40°C for at least 30 min to allow the formation of heteroduplexes. Subsequent pooled samples were then prepared.

One microliter of triple loading dye (AT Biochem) was added to each DNA pool, all of which was loaded onto a 0.8-mm-thick, 30 × 40-cm gel consisting of 0.8× MDE in 0.6× TBE buffer (50 mM Tris-borate, pH 8.3, 1.0 mM EDTA). The gels were electrophoresed at 10 W for 1 h between each loading, then at 4.5–5.5 W (depending on the size of the PCR product) overnight at room temperature in a Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies). Five sequential loadings allow the simultaneous analysis of up to 300 PCRs. Our conditions

Figure 1. A section of a silver-stained heteroduplex gel demonstrating several mutations in exon 41 of the NF1 gene. The 373-bp products were run on a 0.8× MDE gel. Exon 41 is unusual in that some mutations (lanes 2 and 7) produce heteroduplex bands more than 1 cm higher than the homoduplex. Lane M contains a 1-kb ladder; lane 2 (load V), 7268delCA, premature truncation at residue 2424; lane 7 (load V), 7267+A, premature truncation at residue 2425; lane 12 (load I), 7313+A, premature truncation at residue 2443; lane 15 (load I), C→T transition 22 bases proximal to exon 41.
were arrived at after first trying the conditions recommended (2) and using available equipment; however, other conditions may work equally successfully (for example, 0.4-mm-thick gels, more than two DNA samples per pool or ethidium bromide staining).

Xylene cyanol blue dye (incorporated into the triple loading dye) co-migrates with a 230-bp fragment (AT Biochem), giving a good guide. Following electrophoresis, the glass plates were separated and the gel left attached to one plate to avoid its expansion in water. The gel was carefully silver-stained (AT Biochem, personal communication, 1994), then dried onto filter paper (Whatman International, Maidstone, England, UK).

The amount of DNA in each pool is critical; too little and the resulting weak mutant heteroduplex bands can be missed; too much and the excess DNA can easily mask a mutant band with similar mobility to the wild-type band. Therefore, it is essential to carefully observe the appearance of heteroduplex bands throughout the developing stage (sodium carbonate) of silver staining and have the fixing solution ready to prevent further staining.

In DNA pools that produced a mutant band, the two PCR products were subsequently electrophoresed separately to determine which one had the mutation. All mutations were characterized as described previously (16).

The smallest fragment screened, a 190-bp product, was visualized 2 cm below the xylene cyanol blue dye, while the largest fragment, at 549 bp, migrated to approximately 10 cm above the xylene cyanol blue dye. Provided that the homoduplexes migrate at least 30 cm through the gel, the load number does not appear to affect the detection rate. Mutant DNA usually generates one heteroduplex band that migrates more slowly than its corresponding homoduplex however, several mutations produced 2 or even 3 extra bands, some of which occasionally electrophoresed faster than the homoduplex band. Generally, the mutant band belonged to the nearest heteroduplex, however, on a couple of occasions it did not, and both DNA samples from the next nearest heteroduplex were reanalyzed.

Using this technique, we have so far screened 16 exons and characterized mutations in 60 of 480 NF1 patients (48 different mutations consisting of 18 deletions or insertions and 30 substitutions). Several primer sets were unsuitable for this method, producing a smear, or several bands instead of one band; a problem that was usually overcome by redesigning the primers. Polymorphisms are also detected by this method, making the identification of mutant bands more difficult; again this problem can be overcome in the case of intronic
polymorphisms by redesigning the primers to exclude the polymorphic site.
In addition to the above mutations, 21 positive controls previously detected by SSCP (9 deletions, 1 insertion and 11 substitutions) were all detected by HA. However, 2 out of 20 mutations identified by HA were not detected by SSCP (both of which were substitutions).

Although more than 300 mutations have been reported to the National Neurofibromatosis Foundation Inc. (NNFF) International NF1 Genetic Analysis Consortium (7), the mutations in most NF1 patients are still undefined. The technique described here can be applied to any genetic disorder once the causative gene has been cloned, but can prove particularly useful in disease genes with a heterogeneous distribution of mutations throughout the gene.

“Heteroduplex bands” form as a result of mismatched bases (18), reflecting a difference in nucleotide composition (19). Nucleotide variation can result in different conformers that migrate differentially in electric fields, with the more compact DNA migrating faster. This explains the appearance of some of the heteroduplex bands observed migrating faster than the wild-type DNA. If several bands are observed, they might represent each possible combination of DNA present in that particular heteroduplex (i.e., normal-normal, normal-mutant and mutant-mutant). Additional bands can be seen if both samples in the DNA pool contain a mutation.

Lack of heteroduplex formation cannot exclude a mutation in that exon, as this technique, like most others, is not 100% sensitive. The aim of a robust screening protocol is to find most mutations rapidly so that unidentified mutations can be screened using a more sensitive mutation detection technique.

Multiple loadings have also been used to increase the throughput of SSCP and microsatellite markers in our laboratory. We were the first group to use multiple loadings in HA (10,16,17) and SSCP, and to our knowledge, this is the first detailed report of the improved technique. This successful approach has enabled the simultaneous analysis of 300 samples on one heteroduplex gel, clearly increasing throughput and reducing screening time.

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REFERENCES


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