

The rapid detection of unknown mutations in nucleic acids

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The task of identifying mutations in nucleic acid sequences is a vital component of research in mammalian genetics. With the advent of the polymerase chain reaction, several useful mutation detection techniques have evolved in recent years. The different methods have complementing strengths and a suitable procedure for virtually any experimental situation is now available.

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Techniques for the detection of naturally occurring mutations that disrupt genes are of use to biologists in many fields. In human genetics, these methods are used to determine whether a candidate gene is causally related to a phenotype and also to identify new alleles at known loci for diagnostic, population genetics and structure/function studies. Procedures for mutation detection can be separated into two distinct groups. The first consists of techniques which efficiently identify known disease alleles, such as population screening for carriers of the common cystic fibrosis (CF) mutations^{1,2}. The second group consists of methods to scan sequences for unknown mutations and will be the subject of this review. While several useful technologies for the detection of sequence heterogeneity exist, no single method is applicable for all situations. The most appropriate screening technology is influenced by the expected nature of the mutation, size and structure of the locus in question, availability of mRNA, degree of sensitivity required and resources available.

The spectrum of mutations ranges from cytogenetically visible chromosome rearrangements to micro-deletions and insertions and finally single base alterations. Some loci, such as the steroid sulphatase gene³, are very prone to deletions, whereas others, such as the CF gene⁴, are associated with hundreds of point mutations. Candidate loci can be as complex in structure as the dystrophin gene, with a 14 kilobase (kb) peptide coding region and 80 exons⁵ or as simple as the SRY (male sex determination) locus with only one large exon⁶. In recessive autosomal and X-linked diseases the use of mRNA enables larger stretches of coding sequence to be examined together. Many genes of interest, however, may not be expressed in easily accessible samples such as blood or fibroblasts,

leaving analysis of genomic DNA as the only option. Finally, automation and fluorescence detection technology have many advantages, but the high cost limits their availability.

Detection of large gene alterations

Large gene alterations can be contrasted to single base changes and are mutations in which substantial portions (> 500 basepairs (bp)) of a gene are deleted, duplicated or otherwise rearranged.

Cytogenetic techniques

Extremely large (> 1 megabase (Mb)) deletions and insertions can be detected by high resolution cytogenetics⁷. Recently, the power of cytogenetic analysis has been enhanced by the use of fluorescent *in situ* hybridization (FISH)^{8,9}. FISH uses fluorescently labelled DNA probes, which are hybridized to chromosome spreads, to detect not only the presence or absence of a chromosomal region corresponding to the probe, but also its position in the genome. Multiple probes labeled with different fluorescent dyes can be hybridized and analyzed simultaneously. FISH is particularly suited for the detection of aneuploidy, micro-deletions or duplications and complex rearrangements, and has found a wide range of applications ranging from the detection of loss of heterozygosity in tumour samples¹⁰, microdeletion syndromes¹¹ or duplications¹².

Southern blot hybridization

The Southern blot¹³ remains one of the fastest methods to screen quickly for mutations, and offers a good first step in mutation analysis. The main advantage of the technique

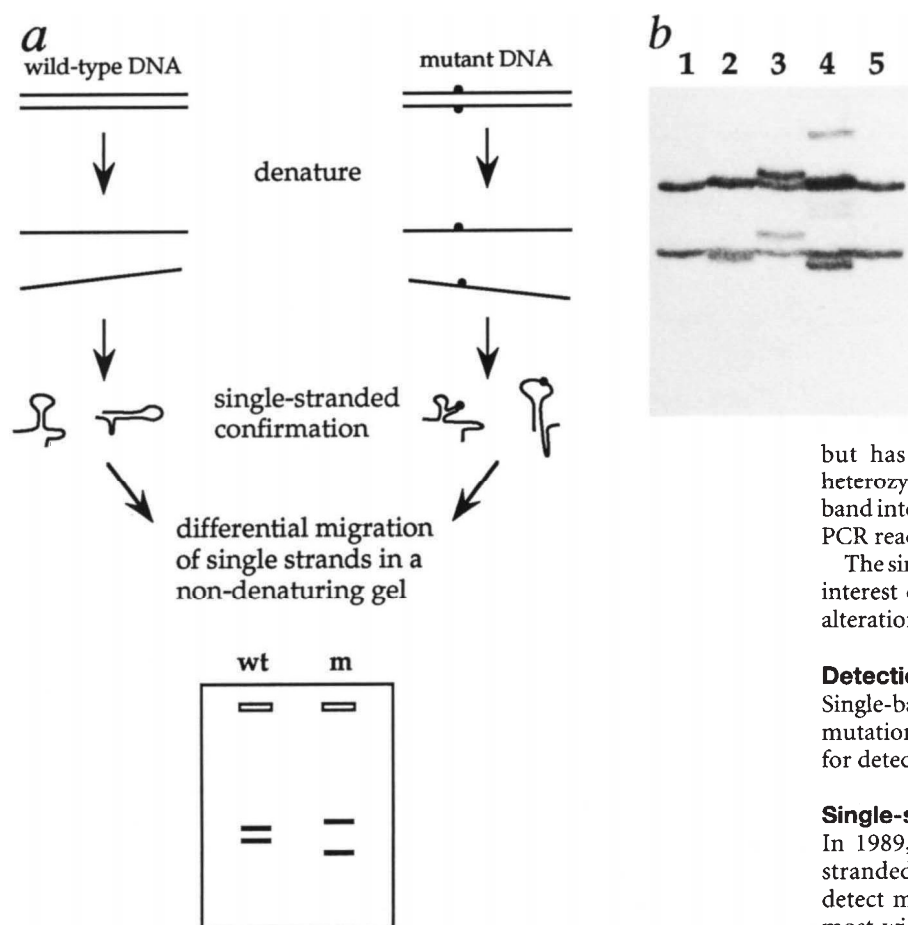


Fig. 1 The principle of SSC analysis. **a**, Schematic representation. Wild-type (wt) and mutant (m) PCR products are shown at the top. The presence of a point mutation is represented by a dot on the DNA strands. Different single-stranded conformation lead to differential mobility in the gel. **b**, SSC analysis of exon 4 of the ornithine aminotransferase gene²⁶. Lanes 1 and 5, normal controls; lanes 2–4, demonstrate band shifts indicating the presence of mutations.

but has been employed for other disorders^{20,21}. In heterozygotes, deletions are seen as 50% reductions of band intensities in a quantitative analysis of the multiplex PCR reaction²².

The simultaneous amplification of several sequences of interest can also be useful in the search for single-base alterations (see below).

Detection of single base changes

Single-base alterations are the most common type of mutation at most loci. A number of methods may be used for detecting these subtle changes.

Single-stranded conformational (SSC) analysis

In 1989, Orita *et al.* first reported the use of single-stranded conformational polymorphisms (SSCP) to detect mutations²³. SSC analysis has since become the most widely used of the scanning technologies. Wild-type and mutant target DNA are amplified by PCR, denatured and then electrophoresed side by side through a (usually glycerol containing) non-denaturing polyacrylamide gel. The two single-stranded DNA molecules from each denatured PCR product assume a three-dimensional conformation which is dependent on the primary sequence. If a sequence difference (mutation) exists between wild-type and mutant DNA, this may result in differential migration of one or both of the mutant strands (Fig. 1). PCR products with altered migration patterns can then be analysed by DNA sequencing to determine the exact nature of the alteration. The main advantage of SSC analysis is its simplicity and relative sensitivity. No additional steps are required after PCR and the gel system is relatively straightforward. The method also lends itself to the simultaneous examination of multiple samples. In most published studies the amplification products are rendered radioactive by the addition of ³²P or ³⁵S labelled dCTP to the PCR reaction. However, non-radioactive detection by silver-staining²⁴ and ethidium bromide staining²⁵ have been successfully used. SSC analysis detects mutations but does not localize them within the fragment.

SSC analysis detects 70–95% of mutations in PCR products of 200 bp or less^{26,27}. The sensitivity of the method decreases with the size of the PCR product and is less than 50% when fragments of >400 bp are analysed. Some of the size limitations of the method may be overcome by the simultaneous amplification and analysis of several fragments in one lane (multiplexing) or by restriction digestion of a larger amplification product prior to electrophoresis²⁸. The use of RNA generated by *in*

is that no detailed knowledge of the structure and sequence of a gene is required and a preliminary screen can be carried out with a probe of interest immediately after its isolation. Large deletions and insertions may be detected by the presence of junction fragments or changes in band intensities in blots (assuming equal amounts of DNA are present in all lanes). Point mutations may also be detectable, if they alter restriction sites. The restriction endonucleases *MspI* and *TaqI* have 4 base recognition sites (CCGG and TCGA respectively) that contain the mutation-prone dinucleotide CpG¹⁴. These enzymes are therefore especially useful in scanning for mutations by Southern blot.

PCR-based detection methods

The ability to amplify DNA enzymatically via the polymerase chain reaction¹⁵ represents the most important advance in mutation detection technology since the development of DNA sequencing^{16,17}. All of the procedures described below rely on PCR amplification of sample DNA prior to analysis.

Multiplex PCR for the detection of deletions

If a locus of interest is prone to deletions and if its genomic sequences are known, the simultaneous PCR amplification of several sequences throughout the gene (multiplex amplification) is the most rapid and practical method for their detection. Deletions are indicated by the absence of some of the bands in the multiplex pattern. This method has found wide use in the diagnosis of Duchenne muscular dystrophy¹⁸, in which 60% of cases represent deletions¹⁹,

vitro transcription of PCR products also appears to improve detection of mutations in larger fragments²⁹.

Denaturing gradient gel electrophoresis (DGGE)

DGGE also relies on differential electrophoretic migration of wild-type and mutant DNA for the detection of mutations. Double-stranded DNA (dsDNA) generated by PCR is electrophoresed through a gradient of increasing concentration of a denaturing agent (urea and formamide). As the DNA migrates, the strands progressively dissociate in discrete sequence-dependent domains of low melting temperature. This partial "melting" of the dsDNA leads to an abrupt decrease of mobility (Fig. 2). DNA molecules differing by only a single base substitution have been shown to exhibit differential mobility in such gels³⁰. The sensitivity of DGGE is greatly enhanced, however, if heteroduplex DNA between wild-type and mutant sequences is used for the analysis³¹. Mutations can be found most reliably when the sequence heterogeneity lies within a domain of relatively low melting temperature. This can be achieved for virtually any sequence of interest by the use of computer programs to predict theoretical melting profiles and design PCR primers³². The attachment of a (30–50 bp) high melting temperature GC-clamp^{31,33} to one PCR primer is critical to ensure that the amplified

sequence has a low dissociation temperature.

Once the appropriate PCR primers and denaturant conditions have been developed for a specific region, DGGE is a highly reliable and rapid method for mutation detection. Single base differences can be detected with ~95% accuracy in PCR products of up to 600 bp in length. Detection is usually carried out by non-radioactive means^{34–36}. As in SSC analysis, while the presence of a sequence difference is detected, its location within the fragment remains unknown and has to be determined by sequencing.

The denaturing gradient can also be generated by temperature and this method is termed temperature gradient gel electrophoresis (TGGE)³⁷.

Heteroduplex analysis (HA)

If mutant and wild-type template sequences are present simultaneously in a PCR reaction, heteroduplexes between the two different DNA species can be formed during the late cycles. Heteroduplex molecules with a single bp variance may show differential mobility from homoduplexes in regular polyacrylamide gels³⁸. This phenomenon is thought to be caused by sequence dependent conformational changes in the dsDNA. Recently, new gel matrices (Hydrolink® and MDE™ from AT Biochem) have become available which markedly enhance the ability to detect mutation induced mobility shifts in heteroduplex molecules. These gel matrices are poured between glass-plates, analogous to polyacrylamide gels used for DNA sequencing. Mutant and wild-type PCR products are electrophoresed side by side and their mobility compared. Recent studies indicate a level of sensitivity similar to SSC analysis (80–90%) in small DNA fragments (<300 bp)^{39,40}. Both isotopic⁴⁰ and non-isotopic³⁹ detection methods have been reported. This technique is attractive because of its simplicity and has been applied successfully to the study of a number of human genetic disorders^{41–43}.

RNase A cleavage

The principal of heteroduplex mismatch analysis applicable to both RNase and chemical mismatch cleavage (see below) is illustrated in Fig. 3. In RNase A cleavage, an RNA-DNA heteroduplex between a radioactive wild-type riboprobe and mutant DNA, generated by PCR, is subjected to cleavage by RNase A^{44–46}. This enzyme will recognize and cleave single-stranded RNA at the points of mismatch. The reaction is analysed by electrophoresis and autoradiography. The presence and location of a mutation are indicated by a cleavage band of a given size. This method is limited by the need for radioactively labelled RNA and the fact that RNase A can only detect ~50% of mismatches. It has therefore been largely replaced by the similar technique chemical mismatch cleavage.

Chemical mismatch cleavage (CMC)

In CMC, a heteroduplex between a radiolabeled wild-type DNA molecule and mutant DNA (or RNA) is created by boiling and reannealing. Maxam-Gilbert sequencing chemistry¹⁷ is then used to chemically modify mismatched bases at the sites of mutations in the DNA-DNA or DNA-RNA heteroduplexes. The labelled DNA is cleaved by piperidine at the site of the modification, and this is followed by denaturing PAGE and autoradiography⁴⁷. Osmium tetroxide is used for the modification of mispaired

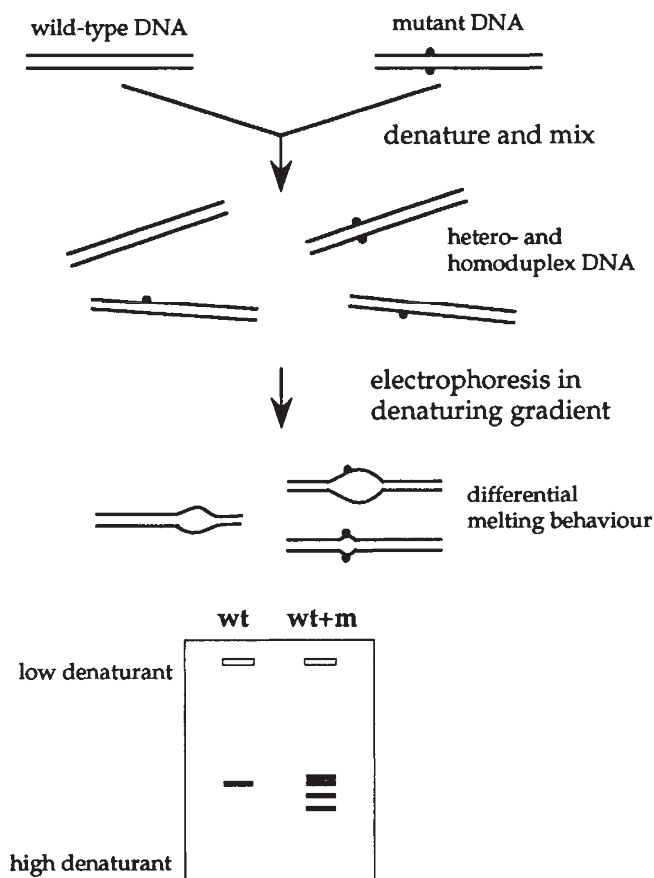


Fig. 2 The principal of DGGE. Wild-type (wt) and mutant (m) PCR products are denatured and reannealed to generate 4 species of hetero- and homoduplexes. Differential melting behavior leads to altered migration in a gradient of denaturing agents compared to wild-type homoduplex.

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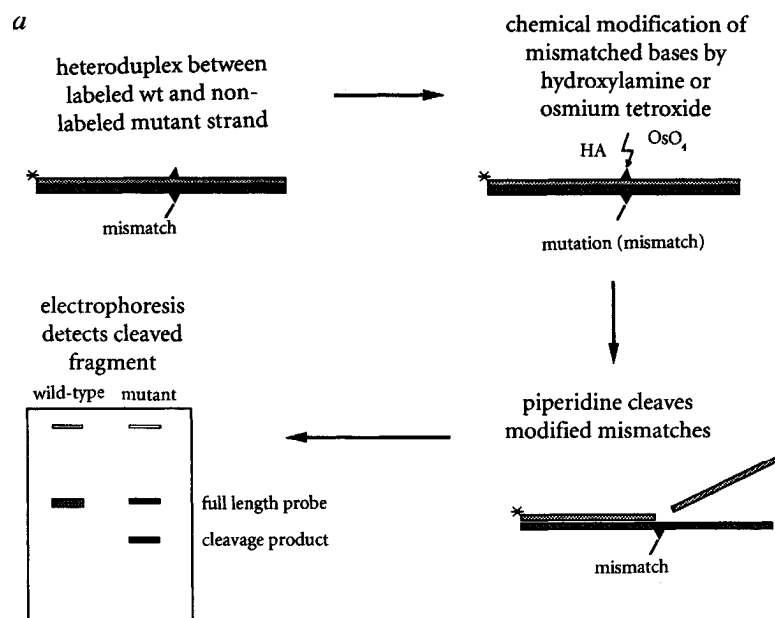


Fig. 3 The principal of heteroduplex mismatch cleavage. **a**, Schematic representation of chemical mismatch cleavage. A heteroduplex between labeled wild-type DNA (top strand) and unlabeled mutant DNA is shown. The mismatched base at the site of the mutation is chemically modified and subsequently cleaved by piperidine. **b**, CMC analysis of the human ornithine transcarbamylase cDNA⁷⁰. Cleavage products indicating the presence of mutations can be seen in all lanes except lane 4, which represents a normal control.

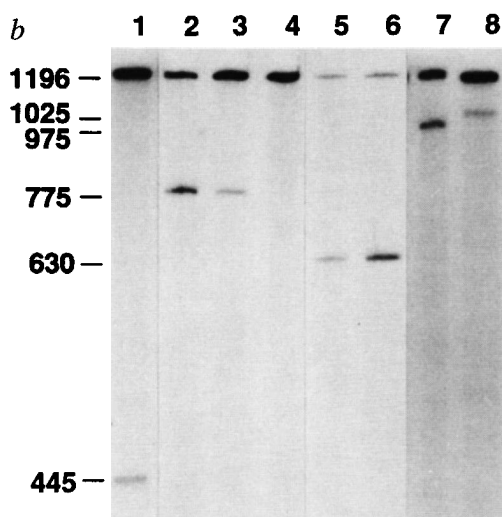


Table 1 Advantages and disadvantages of mutation scanning methods

	Size	Sensitivity	Localizes	Toxic chemicals	Exon scanning	mRNA scanning	Best ref.
SSCP	250	80%	no	none	+++	+	26
DGGE	600	95%	no	formamide	++	++	67
CMC	1700	>95%	yes	yes	+	+++	68
PCR DS	500	>99%	yes	none	++	++	69
HA	300	80%	no	none	++	+	40

The size of fragments which can be analysed is given in bp. Sensitivity, the approximate % of mutations detectable with the method is given; localizes, indicates whether a method provides the exact location of the mutation within the examined fragment; +++, indicates a high, ++ moderate and + limited usefulness of the method for this application.

thymines and hydroxylamine for mismatched cytosines. Adenosine and guanosine mismatches of the wild-type sense strand are detected by also labelling the anti-sense strand of wild-type DNA in the heteroduplex. CMC is very sensitive, detecting >95% of mismatches when only the wild-type DNA is labelled and 100%, when both wild-type and mutant DNA are labelled⁴⁸. CMC has the lowest size constraint of all the mutation scanning methods. PCR products of up to 1.7 kb in length have been examined successfully⁴⁹, permitting efficient screening of amplified mRNAs. As well as detecting the sequence alteration reliably, the precise localization and nature of the change is also indicated by the size of the cleavage band and the cleaving reagent. Non-isotopic labelling techniques⁵⁰ and multiplexing⁵¹ have been reported for this technique.

A highly sensitive variation of CMC has been described by Ganguly and Prockop⁵². Chemical modification of the mismatched base(s) is carried out with carbodiimide (CDI), and this is followed by primer extension using a radiolabeled oligonucleotide. *Taq* polymerase terminates extension at modified bases and the shortened extension product is detected by autoradiography. This method has been applied primarily for the detection of mutations in collagen genes.

Direct sequencing

All the methods described above are capable of detecting mutations with varying efficiencies, but none defines precisely the nature of the change. DNA sequencing defines the location and nature of the change and therefore is a necessary final step of any mutation detection method. If DNA sequencing can be made rapid, accurate and efficient, it represents the ideal mutation "scanning" technique. Direct sequencing (DS) refers to the direct sequence analysis of PCR products without prior subcloning into sequencing vectors and can be the primary method of mutation detection. As this method becomes more efficient with the use of automation and new fluorescence detection technology, it is likely to become the primary method of mutation detection. Direct sequencing is based on the Sanger dideoxy chain termination method¹⁶, and both isotopic as well as fluorescence detection techniques have been described. Fluorescence based sequencing methods have many advantages, but require expensive specialized equipment.

In order to sequence PCR products successfully by the conventional dideoxy termination protocol, it is essential to convert the double-stranded PCR product into a single-stranded sequencing template. Several methods have been described to achieve this. In the first technique, termed asymmetric PCR⁵³, the PCR product is reamplified in a second reaction in which one of the oligonucleotide primers is in >100:1 excess of the other. This creates an abundance of single-stranded PCR product for sequencing. In the second method one of the PCR primers is biotinylated. After the reaction, the double-stranded PCR product is captured on an avidin-coated magnetic bead^{20,54}. The non-biotinylated strand is melted away with NaOH and the sequencing reactions are carried out on the immobilized single-stranded template. In a third approach, termed "genomic amplification with transcript sequencing" (GAWTS) the original PCR primers carry T7 RNA polymerase binding sites⁵⁵. *In vitro* transcription is then used to generate single-stranded RNA template for sequencing.

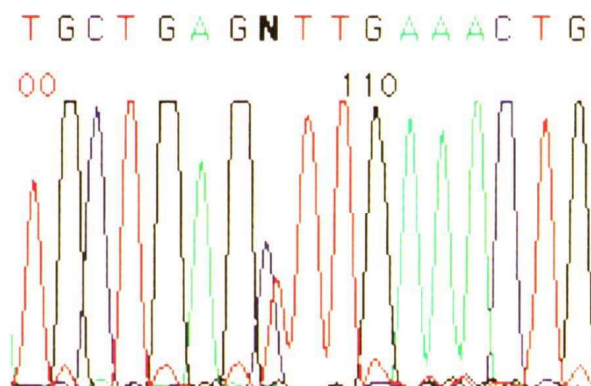


Fig. 4 Direct sequencing with fluorescent primers. A portion of the human CD4 genomic locus was sequenced. In the position marked by an N, a G and T peak appear in the same position indicating a sequence alteration on one chromosome.

A recent promising modification of dideoxy chain termination sequencing technology is cycle sequencing, also termed amplification sequencing^{56,57}. In this method, the template is simultaneously amplified and sequenced by the addition of dideoxy terminators to a PCR reaction. This technique has the advantage of requiring only minute amounts of template. When isotopic detection methods or conventional fluorescence sequencing protocols are used, however, cycle sequencing requires that the sequencing primer be end-labeled (either by an isotope or by a fluorochrome). Recently, a new protocol based on cycle sequencing and fluorescence detection technology has been developed⁵⁸. Fluorescently labeled dideoxy terminators (dye terminators) are used with a different fluorescent dye coupled to each of the four ddNTPs. The extension product is labelled at the site of the dideoxy termination rather than at the sequencing primer end. In this direct sequencing protocol, neither specially modified PCR or sequencing primers are needed.

An example of a result obtained by fluorescent technology direct sequencing is shown in Fig. 4.

Future methods

The pace of improvements in mutation detection

techniques over the past few years has been rapid, and new and promising advances are being tested in many laboratories. Three of these new methods will be mentioned here. The first is termed dideoxy fingerprinting (ddF). ddF represents a combination of SSC analysis and direct sequencing⁵⁹. After amplification, the PCR product is sequenced with dideoxy CTP (ddCTP) to generate a "C-ladder" (fingerprint) of bands. The sequencing reactions from wild-type and mutant samples are then electrophoresed through a SSC (non-denaturing polyacrylamide) gel. Mutations are detectable as shifts of individual bands in the ladder. This method, when applied to the factor IX gene, was highly sensitive (84/84 alleles detected) and also provided some information about the localization of the sequence alteration.

The second new technique utilizes a computer software variation in the analysis of fluorescent cycle sequencing data⁶⁰. Fluorescent sequencing is carried out in routine fashion, and all 4 termination reactions are loaded into a single gel lane. A set of 4 adjacent lanes is then used for the parallel analysis of the sequence ladder generated by a single dideoxy terminator (single color analysis). This permits the detection of additional peaks which indicate mutations. Extra peaks are detectable if up to 4 individual sequencing reactions are loaded into a single lane, thus permitting the simultaneous analysis of 16 samples. This method appears especially useful when high throughput automated sequencing is available.

Recently, *E. coli* mismatch repair enzymes have been used for the detection of known mutations in oncogenes⁶¹. If these enzymes are able to detect mismatches in larger heteroduplex molecules containing unknown mutations, they may find wide application in mutation detection.

Mutation versus polymorphism

The methods described above will detect nucleic acids alterations but do not necessarily define their biological significance. Some sequence changes, such as those causing frame-shifts or chain terminations, are obviously functionally deleterious to a protein. Other alterations, such as missense mutations leading to amino acid substitutions or base changes in introns and untranslated regions, can be either functionally relevant or represent sequence polymorphisms. Additional information

including family studies, population studies or functional assays after *in vitro* expression may be needed to determine the significance of a sequence alteration.

Comparison of methods

The relative usefulness of the techniques mentioned above for the detection of single base alterations depends upon a number of criteria. Table 1 summarizes the advantages and disadvantages of the different procedures. Obviously, the level of expertise for a certain procedure, and the equipment available, are other determining factors.

Size factors: mutant mRNA versus genomic DNA

The two principal materials on which

Table 2 Genomic DNA versus mRNA as starting material for mutation analysis

	Genomic DNA	mRNA
Advantages	Easily accessible (blood) In autosomal loci both alleles are equally represented Mutations in the promoter and intronic splice junctions can be detected First choice in autosomal dominant traits	Long segments of peptide coding region can be analysed Gene structure information not needed Fewer PCR reactions Aberrant mRNA sizes can be seen First choice in X-linked traits
Disadvantages	Genomic sequence and gene structure information are needed Only small segments of coding region (exons) are analysed More PCR reactions	Gene may not be expressed in accessible specimens In autosomal loci only one allele may be represented Mutations in the promoter and intronic splice junctions are not detected

mutation analysis can be carried out are the mRNA and genomic DNA. Several considerations are important in deciding which material to use for mutation detection (Table 2). If mRNA is readily available (from cultured cells or pathological specimens), it is usually the most practical starting material because larger stretches of coding sequence can be scanned. The use of specialized PCR protocols may permit the amplification of mRNA from tissues in which the gene is expressed at only very low levels (illegitimate transcription)^{62, 63}. The mode of inheritance of a phenotype for which mutations are being sought also has an impact on the choice of starting material. mRNA analysis is less desirable for dominant conditions, because the mutant allele may not be represented in mRNA (null alleles).

In mRNA analysis, reverse transcription is used to generate first strand cDNA, and this is followed by PCR amplification to generate large (1–2 kb) fragments from cDNA⁶⁴. When genomic DNA is used, smaller segments of coding sequence (exons) usually are amplified. The different mutation detection techniques are not equally useful for both approaches. SSC and HA are particularly advantageous for the study of small DNA fragments (<300 bp), notably in the exon analysis of genomic DNA. Direct sequencing and DGGE are sensitive up to 500–600 bp, and CMC can be used for fragments up to 1700 bp.

The methods capable of scanning large segments of DNA are also useful in the study of organisms with few introns such as bacteria and viruses^{65,66}.

Sensitivity and accuracy

As mentioned above, the sensitivity of the different mutation detection procedures is dependent on the size of the fragment to be analysed. Even when scanning small DNA fragments, however, SSC analysis and HA are not as sensitive as DGGE, CMC and direct sequencing. Sequencing and CMC are the only methods that accurately localize the mutation within the examined fragment.

Candidate gene versus known defective gene

If a gene is known to be defective (in a patient with a genetic disease), the requirements in mutation analysis are different than when a gene is a candidate, but not already known to carry a mutation. Known defective

genes are often examined in a diagnostic setting, in which a high degree of accuracy is needed. Frequently the same locus is analysed repetitively in large numbers of patients or in populations for carrier detection. In these situations, it may become practical to design specialized oligonucleotides for direct sequencing (biotinylated or fluorescent) or DGGE (GC-clamps). Direct sequencing and DGGE are more sensitive than SSC analysis or heteroduplex analysis and are therefore frequently used in this setting, even for the testing of small DNA fragments. The specialized set-up required is worthwhile because of the large sample numbers and because of the degree of accuracy required. This is in contrast to a candidate gene analysis, in which any given locus may or may not have mutations. Specialized reagents and equipment are less practical in this scenario, and it is less critical to detect 100% of mutations, if enough patient samples are available. Often details of the structure of the candidate locus are not known and examination of mRNA (only coding sequence needed) is desirable. In this context, the size of the sequence to be examined usually determines the method of choice (see above).

Radioactivity and toxicity

In all procedures described, the detection methodology can be either radioactive or non-radioactive. SSC, DGGE and heteroduplex analysis can be easily carried out non-isotopically with simple ethidium bromide staining of DNA. This is less simple for CMC and direct sequencing, for which fluorescence detection is needed. Of all the techniques, CMC clearly has the most problems with the use of toxic chemicals. Both osmium tetroxide and piperidine are highly noxious and not suitable for use in a clinical laboratory environment.

Simplicity and speed

SSCP, HA and DGGE (once set up) are clear front runners for simplicity, in that the analysis of the PCR product can be carried out immediately after the reaction. CMC and sequencing both require several manipulations after PCR and are more time-consuming. Automation and dye terminator cycle-sequencing, however, are making direct sequencing a very rapid approach.

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