

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 ± 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- μ) 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	5×10^6	Metaphase spread is used as a probe for test and control differential hybridization (84)
FISH	5×10^4	Modifications (e.g., Fibre FISH) to improve resolution (85)
Array CGH	5×10^4	Uses arrayed BACs instead of chromosome spreads (86)
MAPH	1×10^2	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	1×10^2	Requires careful control of starting DNA concentration and quality. Gives relative concentrations; thus is semi-quantitative (88)
Competitive PCR	1×10^2	Extremely accurate, provided the competitor is accurately dispensed. Gives molar quantities; thus is absolutely quantitative (89)
Real-time PCR	1×10^2	More expensive to set up; various detection methods available, including SYBR Green or fluorescent probes in TaqMan or Beacon format (90)
Long-PCR	1×10^2	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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