Advances in approaches to DNA-based diagnostics

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The most tangible advances in DNA diagnostics during the past year have been in enhancing existing techniques to simplify their use and improve throughput. This has led to simplified genotyping methods using homogeneous analysis coupled with spectral data output. Miniaturisation and increased throughput have also been achieved through improvements in DNA chip technology.

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Abbreviations

ARMS	amplification refractory mutation system
FRET	fluorescence resonance energy transfer
HANDS	homo-tag assisted non-dimer system
OLA	oligonucleotide ligation assay
PNA	peptide nucleic acid
RRS	resonance Raman scattering

Introduction

DNA-based diagnosis is the application of molecular techniques to identify the various clinically relevant categories of genetic changes. To be truly useful, the diagnostic must identify the appropriate changes accurately and it must do so in a convenient and practical way. The choice of method will be influenced by the actual clinical application but it should be sensitive, specific, simple, quick, and cost-effective to perform. For these reasons the various approaches must be considered in the light of both their eventual application and the environment in which they will be applied.

In this review, we focus on molecular techniques rather than cytogenetic methods. We consider specifically the issues concerning the analysis of human genetic variation in the clinical setting. We have not considered methods for detecting gene copy number differences, length polymorphisms or variations in gene expression. Neither have we considered the detection of, or discrimination between, infectious disease agents, although many of the techniques discussed here are applicable to such analytical needs.

Clinically important human genetic variation is most frequently observed as point mutations or other small DNA variations that alter gene function. It is these micro genetic variations that genetic tests must detect accurately. Depending on the specific clinical application there are large differences in both the number of point mutations or polymorphisms that are relevant to the analysis and the level at which they are present in the sample. The number of point mutations needed for a particular diagnostic test varies from one (e.g. sickle cell anaemia) to perhaps up to 10,000 (e.g. the hereditary breast and ovarian cancer gene BRCA1 analysis). For inherited mutations and polymorphisms, there are always two, one or no copies of the mutation in each diploid cell. Cancer cells, which carry acquired mutations, however, may be mixed with a large excess of normal cells. The level of this type of mutation within the total cell population can vary, therefore, from 100% to less than 0.1%. The ratio of mutant:normal DNA can also differ in conditions involving mitochondrial genomes, and here the level of mutation in the sample can vary in the 5–95% range. From these considerations and those above, it follows that for maximum efficacy the choice of a point mutation analysis system should be dependent on the specific clinical need.

Methods that do not amplify the analyte

Within a typical sample for a DNA-based diagnostic test the numbers of target molecules may range from ten to 10^5. Traditionally, a target amplification method has been employed to increase the number of molecules to a detectable level. There is, however, much interest in eliminating the need for an amplification step altogether by using hypersensitive detection techniques. These techniques, in principle, would be faster and simpler to perform because the dominant target amplification step is removed, as is the issue of cross contamination of unamplified analytes with products of amplified analytes. The single most practical challenge for such techniques is that there are often limited numbers of molecules of genomic DNA present in the sample; a situation which is exacerbated when it may be necessary to analyse for multiple DNA variations. One solution to this problem is to amplify the signal as opposed to the target DNA. Branched DNA (one branched DNA or dendrimer can hybridise to the target which then provides multiple hybridisation sites for the detection probe) [1–4] and dendrimers [5] have been applied successfully to problems of detection of infectious agents where as few as 50 molecules/ml or 10^{-19} M are detectable.

Alternative methods that do not rely on either amplification of the analyte or the signal may also be sufficiently sensitive for use in the clinical setting. Surface-enhanced resonance Raman scattering [6••] brings together two previously established technologies, resonance Raman scattering (RRS) and surface-enhanced Raman scattering. RRS occurs when light incident on a dye is scattered due to the excitation of electrons, which results in excited electrons going to vibrational energy levels different from that prior to excitation. The

spectral lines from RRS are enhanced by surface-enhanced Raman scattering, thought to result from higher valence electrons of the excited analyte associating with pools of electrons, known as 'plasmons', in the pits of a roughened metal surface. The operating principle of surface-enhanced resonance Raman scattering for nucleic acid detection relies on the addition of a chromophore to the nucleic acid probe, adsorbing the conjugate to colloidal silver and irradiating this with laser light tuned to the resonant frequency of the chromophore. The spectral information gained allows the identification of different individual chromophores either separately or in mixtures with detection down to single molecule levels. Fluorescence correlation spectroscopy is also capable of detecting single molecules in solution [7]. Fluorescence correlation spectroscopy records spatio-temporal correlations among fluctuating light signals (the motility of the fluorophores in solution is related to the size and shape of the molecules), coupled with devices for trapping single molecules in an electric field. It allows fast screening of spectra in which targets are labelled by specific fluorescent ligands and can monitor concentrations down to 10^{-15} M. The final technique within the non-amplification category is single-molecule electrophoresis [8] for the detection and identification of single molecules in solution. Single-molecule electrophoresis involves the determination of electrophoretic velocities by measuring the time required for individual fluorescently tagged molecules to travel a fixed distance between two laser beams. With appropriate controls the technique indicates changes in base composition.

The application of any of these technologies that do not amplify the target DNA to human genetic analysis will require a solution to the problem of human genome complexity. Any such solution will need to provide a means of reducing background signals derived from non-specific hybridisation elsewhere within the 3×10^9 bp haploid human genome.

Target amplification techniques

PCR remains the dominant technique amongst those that amplify the target DNA. Others, for example, strand displacement amplification [9,10] and nucleic acid sequence-based amplification [11], have not found widespread application outside of their originating laboratory. PCR itself has been systematically improved in many ways. One of the first improvements was the adaptation for use in an allele-specific manner (one allele is amplified specifically and so ends up being in excess of the other alleles, giving a good signal:noise ratio), this development, the amplification refractory mutation system (ARMS) [12], transformed PCR to permit genotyping of DNA directly without the need to probe PCR products after the reaction. Further improvements have been in the instrumentation, which have allowed the reaction volume and time to be reduced significantly. PCR has been adapted to run in a continuous flow system on a chip [13••] described as a micromachined chemical amplifier that moves the sample through thermostated temperature zones on a glass microchip. Using this technique, reaction times have been reduced to as little as 90 seconds. An analogous system operating in real time uses silicon-based, high-efficiency reaction chambers with integrated heaters [14,15••]. Optical windows in the silicon and solid-state diode-based detection components of the instrument perform real-time fluorescence monitoring of the product DNA. Amplification is again very fast and the system has been used in conjunction with TaqMan probes [16] for fluorescent single-base polymorphism detection [15••]. Cheng *et al.* [17] describe the use of similar silicon-glass microchips where PCR was performed directly on isolated cells with detection by capillary electrophoresis.

Analyte amplification techniques coupled with advances in mutation discrimination

Further development of existing methods has given rise to practical and robust diagnostic techniques that are most commonly based on one of four principles.

Primer extension

ARMS [12], can be considered a 'molecular switch' whereby the extension only occurs if the primer is matched to the template DNA at its 3′-end. Because an ARMS primer must detect a specific DNA sequence before amplification can proceed, as opposed to detection after amplification, the method can detect a mutation in complex genomic DNA even when it is vastly under represented. This makes it one of the methods of choice in the analysis of cancer markers, for a recent example see Fox *et al.* [18•] where the K-*ras* status of over 300 colorectal carcinoma samples was analysed this way. Another important attribute of ARMS is the ability to multiplex many reactions, as multiple loci can be analysed simultaneously in the same reaction. Robertson *et al.* [19] have shown, how with careful optimisation, 12 of the most common mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene can be detected simultaneously in just one two-tube test. Further advancements of ARMS technology are discussed below.

Oligonucleotide ligation

The oligonucleotide ligation assay (OLA) or ligase chain reaction [20] is based on the ability of two oligonucleotides to anneal adjacent to each other on the target DNA molecule. The oligonucleotides are then joined by a DNA ligase only if the nucleotides at the junction are correctly base-paired. Through cycles of melting off and more single molecules annealing and being joined, there is an accumulation of the joined product. When the two separate, but adjacent, probes are labelled changes in fluorescence can be monitored as the joined product accumulates and the unjoined probes are depleted. Homogeneous assay applications of OLA are discussed below.

Hybridisation

Dot blot analysis of PCR has been largely superseded by reverse dot blots, so that many allelic variations can be

characterised simultaneously for a single PCR product derived from an individual clinical sample. The original technique [21] used oligonucleotide probes bound to nylon membranes via homopolymer tails. Hybridisation of PCR product was then detected by the binding of streptavidin-horseradish peroxidase to the biotinylated DNA followed by a colourimetric reaction. The principle of this assay is retained using oligonucleotide array technology on microchips [22–26,27•]. DNA array methods are considered further in the specific context of heterogeneous assays below.

Single nucleotide incorporation

Mini sequencing [28] or single nucleotide primer extension involves the single base extension of an immobilised primer where the incorporated base corresponds to the allelic variation of interest. Recently, the use of all four bases, each labelled with a different fluorophore, allows the scoring of allelic variation according to the fluorescent emission wavelength [29]. Fluorescent analysis coupled with multiplex PCR and DNA chip technology [30] may be a potential candidate for high-throughput mutation detection and genotyping.

Major advances in the formatting of PCR-based assays

PCR-based assays can be carried out in either an homogeneous or a heterogeneous manner. Homogeneous assays are those that require no further manipulation after setting up the reaction. Signal generation and detection are carried out in the unopened reaction vessel once the reaction is complete or continuously during the course of the reaction (i.e. in 'real time'). Heterogeneous assays require further manipulation once the amplification reaction is complete, this may be to aliquot the reaction for a number of analyses to be performed or to add some component of a signal generation process. The number of analyses required from one sample dictates the choice between homogeneous and heterogeneous systems but there are two overriding advantages of homogeneous systems in that amplification product from one reaction cannot carry-over into future reactions and they can be performed in real time.

Homogeneous methods

Homogeneous techniques can be divided between specific and non-specific ones. The first homogeneous technique described monitors fluorescence accumulation that results from intercalation of ethidium bromide into the accumulating PCR product during thermocycling [31,32]. This type of assay falls within the non-specific category because it gives a signal irrespective of the PCR product generated (i.e. it cannot distinguish between a specific or non-specific amplification product, such as primer-dimer). Sunrise probes [33] provide an improvement to non-specific homogeneous assays by using the observation that certain molecules will quench fluorescence when in close physical proximity to a fluorophore, a property known as fluorescence resonance energy transfer (FRET). Sunrise probes are dual labelled with one fluorophore at the 5′-end and another internal. The 3′ region of the probe is target-specific and the 5′ region is self-complementary so that when unextended (i.e. not incorporated into amplicon) it forms a hairpin structure that holds the quencher and reporter fluorophores together. When the probe is extended by incorporation into a double-stranded molecule the fluorophores are held apart by the newly copied complementary strand. This leads to increased fluorescence as amplicon accumulates by not quenching the fluorescence from the reporter. With a variety of fluorophores to choose from, several amplicons can be monitored simultaneously but use in the clinical setting is limited because incorporation into non-specific product or primer-dimer will still give an apparently positive result. Some of these issues can be overcome by the use of appropriate primer design [34] or the use of automated hot-start PCR [35]. Alternatively, primer-dimer accumulation can be suppressed completely using the homo-tag assisted non-dimer system (HANDS) [36]. Each of these approaches improves but does not ensure specificity.

The development of sequence-specific homogeneous methods was a major advance. The first such technique is an approach that monitors increases in anisotropy or fluorescence polarisation as a measure of binding of an amplicon-specific probe to a PCR product [P1]. This technology has been subsequently combined with ARMS to give a homogeneous genomic DNA genotype analysis method [37]. The development of other fluorescent techniques are all based on FRET. The different methods have different ways of making FRET happen as a function of a sequence-specific interaction. TaqMan [16] uses *Taq* DNA polymerase, a 5′–3′ exonucleolytic enzyme with no 3′–5′ exonuclease activity. These properties allow the detection of an amplicon as the PCR proceeds in real-time by including an oligonucleotide (TaqMan) probe designed to hybridise downstream of one of the amplimers. TaqMan probes are blocked from extension at their 3′-terminus and are labelled with a fluorescent reporter at the 5′-terminus and another fluorophore that quenches the fluorescence of the reporter when both are in close proximity. Degradation of the probes from their 5′-end liberates label and so TaqMan specificity results from probes annealing to their amplicon followed by their cleavage that separates the two fluorophores. Developments to TaqMan have been made in the miniaturisation of the technique whereby it is performed in nanoliter volumes in microcapilliaries in a potentially automatable manner [38•]. It has also been reduced to a universal assay so that a single TaqMan probe can be used for the detection of any PCR amplicon (a probe recognition sequence that is not target specific is incorporated into one of the PCR primers for each amplicon and this probe recognition sequence is used in the initial cycles of amplification) or just one pair of probes for genotyping any bi-allelic polymorphism are all that are required [39]. Here

TaqMan was combined not only with ARMS but also with HANDS to give an integrated system for the direct real-time measurement of allele-specific amplicon generation coupled to the suppression of primer-dimer accumulation in PCR. This method, called 'Three-STAR', does not, however, retain the specificity of TaqMan because probe hybridisation is no longer to target-derived DNA. Nevertheless, primer-dimer accumulation, one major source of non-specificity, is suppressed simultaneously by the application of HANDS. Three-STAR was first demonstrated by using the same pair of TaqMan probes in the analysis of three different polymorphic sites in three different genes [39]. Dye-labelled oligonucleotide ligation [40], is a fluorescent adaptation of OLA or ligase chain reaction [20] that combines PCR and OLA in a two-stage thermal cycling sequence with the specific ligation of dye-labelled oligonucleotide probes monitored in real time by measuring FRET. The final FRET-based system, molecular beacons, operates by using hairpin-shaped oligonucleotide probes, each with an internally quenched fluorophore. These probes undergo a conformational change which restores fluorescence when they are hybridised to their specific target [41]. A variety of differently coloured fluorophores are available allowing several molecular beacons to be used to distinguish multiple targets in the same solution [42••,43], thereby providing a means for their use in potentially high-throughput automated 'spectral genotyping' [43].

An alternative to fluorescence-based techniques is chemiluminescence. Nelson *et al.* [44] have demonstrated an in-solution technique based on the hybridisation protection assay that uses oligonucleotide probes labelled with a highly chemiluminescent acridinium ester. Hydrolysis of the acridinium ester destroys chemiluminescence, so that when an acridinium ester-labelled probe hybridises to a complementary target it is protected from hydrolysis. This technique is not truly homogeneous as it requires the post-addition of the probes. Using this principle, all 12 single mismatches analysed were clearly discriminated from their corresponding matched sequences. The advantages of these fluorigenic and chemiluminescent sequence-specific homogeneous methods in a diagnostic context include ease of use, speed to result, no potential for cross-over contamination, low hands-on time, a numerical output, the potential for automation and low cost.

Hybridisation-based heterogeneous methods

When small numbers of mutations or polymorphisms are to be analysed for each sample there are clear advantages in employing sequence-specific homogeneous methods. As the number of variable bases for analysis increases, the attractiveness of homogeneous relative to more traditional techniques, such as sequencing or DNA hybridisation, shifts. One reason for this is the possibility of using physical separation to access many points of information in a small area.

There has been much interest in the development of high-density oligonucleotide arrays, so called DNA chips, which have particular applications in analysing for many potential mutations [22–26,45]. This technology may provide the ability to scan any given gene rapidly to determine the zygosity (i.e. discrimination between homozygotes, heterozygotes and compound heterozygotes) for all possible allelic variations in patient samples. Light-directed chemical synthesis has been used in the construction of DNA chips. This, in conjunction with dedicated instrumentation and software provides for miniaturisation, fluorescence detection, data acquisition and the analysis of hybridisation patterns on the chip showing which polymorphisms are present [22]. To demonstrate this, heterozygous mutations in a 3.45 kilobase exon of BRCA1 were analysed using DNA chips and two-colour fluorescence analysis of reference and test samples [25,26]. In this example, high-density arrays consisting of over 96,600 oligonucleotides 20 nucleotides long were used to screen for a wide range of heterozygous mutations in the BRCA1 gene. Alternative DNA chip technology uses polyacrylamide gel pads that contain allele-specific immobilised oligonucleotides, fixed on glass slides of the microchip. These have been used for hybridisation of fluorescently labelled RNA transcripts of PCR-amplified genomic DNA. The simultaneous measurement in real time of the hybridisation and melting on the entire oligonucleotide array is carried out by fluorescence microscopy [45]. For further review of DNA chip technology see [46].

Use of an oligo array requires four distinct steps: PCR amplification of the regions to be analysed; fractionation; hybridisation in a reverse dot blot format [21] to the array; and analysis of the hybridisation pattern. For diagnostic utility, the time and effort involved in all the stages is an important consideration and this has been explored by Wang *et al.* [47••] in a large-scale study of single-nucleotide polymorphisms. In this survey, 2.3 megabases of human genomic DNA was examined by a combination of gel-based sequencing and high-density DNA array chips. A total of 3,241 candidate polymorphisms were identified and a genetic map was constructed showing the location of 2,227 of these. Genotyping chips were then developed that allowed the simultaneous analysis of 500 polymorphic sites. Clearly, the analysis of 500 loci would not be cost effective if each required an individual PCR reaction as the first step. The key parameter for effective chip application is, therefore, the degree of multiplexing possible that is consistent with accurate mutation detection. This was also investigated in an attempt to reduce the amount of sample preparation required and it was found to be that of 558 polymorphic sites 92% could be detected in 24 sets of 23 loci, 85% in six sets of 92 loci and just 50% in one set of 558 loci could be detected with varying efficiencies at other intervals between the extremes [47••]. While this efficiency may be acceptable in the research context, further

improvements are needed for routine application in the medical diagnostics arena.

An alternative approach to the reverse dot-blot method [21–26,27•,47••] is the forward dot-blot on replicate filters, the so called sequencing by hybridisation format I [48••]. Here, multiple aliquots from individual PCR reactions are dispensed robotically onto membranes in a 384 microwell type array. Individual aliquots are then probed using a single labelled probe for each. Sequencing by hybridisation was demonstrated by the analysis of p53 gene mutations where sequential hybridisation of 8,192 probes to 50 samples was performed. This required more than 400,000 reactions, so clearly the utility of this approach will depend on the level of automation that can be achieved.

A common feature of all multiple hybridisation approaches is that the compromises necessary to allow simultaneous detection of many sequence variants limits the signal:noise ratio of the assay. Whereas the use of redundancy means that these methods are probably sufficient to discriminate heterozygotes, there is a serious limitation of this approach for some aspects of human genetic analysis. Because of small signal:noise ratios, the detection of under-represented mutations is not possible. For example, in the analysis of acquired mutations in certain cancers a specific mutation may only be present in 5% of the cells from which template DNA for a PCR is prepared. Clearly a signal:noise ratio in the region of 50:1 or better would be necessary for that mutation not to be 'missed'. The most appropriate method for this type of analysis is ARMS [12], as demonstrated by Fox *et al.* [18•], because this technique requires detection of a mutation prior to amplification to allow amplification to proceed and so increasing the ratio of mutant to normal DNA for detection. This is contrary to other methods where amplification of all alleles retains the original ratio of mutant to normal DNA at the detection stage. ARMS has been coupled with other advances in heterogeneous detection.

Other heterogeneous methods

Gibson *et al.* [49] describe a rapid colourimetric method for the detection of PCR products. This method provides a sensitive assay for the pyrophosphate ions released through dNTP incorporation as a PCR proceeds, with colour generation monitored by a simple spectrophotometer. The authors describe the use of this assay in conjunction with ARMS analysis to determine accurately a variety of cystic fibrosis and factor V allelic variants [49].

A selective colourimetric detection method based on mercaptoalkyloligonucleotide-modified gold nanoparticle probes has been described by Elghanian *et al.* [50]. Although this was demonstrated using a single-stranded target 30 base oligonucleotide model, the unoptimised system could detect about 10 femtomoles. Hybridisation in a solution containing the appropriate probes produces a polymeric network of nanoparticles and a visibly detectable colour change on drying that allows the differentiation of imperfect targets. Diagnostic application of this technique will require the demonstration that larger PCR amplicons are detectable.

Mass spectrometry is attracting much interest although its application is currently limited to the analysis of small DNA molecules. The application of this technology to DNA diagnostics has been covered extensively in a previous review in this series [46].

Peptide nucleic acid (PNA) is a DNA mimic and thiol-derivatised PNA probes incorporated into a DNA hybridisation biosensor have been used for sensitive determination of single-base mismatches [51]. The specificity of PNA probes on quartz crystal microbalance transducers is attributed to the formation of a PNA monolayer and the use of a hydrophilic ethylene glycol linker. A model that used oligonucleotides corresponding to a polymorphism of the p53 gene system has demonstrated the principle of the technique; however, although this type of device offers rapid and accurate results it has yet to be applied to clinical material.

Conclusion

The clinical requirements and context in which diagnostics are to be applied will be decisive in the selection of which of the recent advances will actually translate into DNA diagnostics. While there is a research capability to test for thousands of polymorphic variations, it is dubious whether this capability will ever be exploited in the clinic where it may be more appropriate to be able to examine larger numbers of samples for fewer variations.

For the future, complexity reduction of the genome to allow direct, unamplified, detection will become a key area of interest. It is also probable that major advances will come from integration of all aspects of DNA analysis from sample preparation through to the result. This could either be as traditional diagnostic work stations or possibly as point of care devices. Although the instrumentation and platforms on which the technology will reside are critical, these aspects are, however, outside the scope of this review.

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