Mutation Detection by Real-Time PCR

K.J. Edwards and J.M.J Logan

Abstract

Real-time PCR is ideally suited for analysis of single nucleotide polymorphisms (SNPs) and has been increasingly used for this purpose since the advent of real-time PCR and as whole genome sequences have become available. It requires methods that are rapid, sensitive, specific and inexpensive, and several real-time methods have evolved which fulfil these requirements. Additionally real-time PCR is a technique that is readily amenable to automation and no post-PCR handling is required. Different formats have been applied including hybridisation probes with melting curve analysis, hydrolysis probes, molecular beacons and scorpion primers. SNP detection by real-time PCR has found applications in diagnosis of human disease, pharmacogenetics, clinical microbiology and drug development, and has replaced techniques such as sequencing, single strand conformation polymorphism and restriction enzyme digestion.
Principles of Mutation Detection

The detection of mutations is a fast growing field of increasing importance for many areas of science including diagnosis of human disease, pharmacogenetics, drug development and microbiology. Mutations are classed as one or more changes in the DNA bases and can include large re-arrangements such as translocations, inversions and gene insertions/deletions or small alterations such as point mutations and base insertions/deletions (SNPs). SNPs are the commonest type of DNA sequence variations and can occur once every 100-300 bases. As the requirement for rapid, reliable, sensitive and inexpensive methods for SNP detection grow the number of techniques available also increases, each with inherent strengths and weaknesses. Most of these techniques can be divided into hybridisation-based or enzyme-based methods and have been extensively reviewed (Syvanen, 2001; Kirk et al., 2002; Kwok, 2002).

Real-time PCR, a hybridisation-based method, has become widely used for mutation detection. The systems are flexible with a number of different probe systems that can be used and there is additional flexibility in the design of the probes. Several formats have evolved including, hybridisation probes, hydrolysis probes, molecular beacons and scorpion primers. These methods are sensitive and specific, inexpensive, rapid (some assays can be performed in as little as 30 minutes) and they are both easy to perform and interpret the results. All of the available platforms are semi-automated and none require additional post-PCR handling for example, agarose gel analysis. Depending on the platform used real-time PCR is suitable for low to medium sample throughput. The greatest advantage of these systems is the quality of the data that is generated, known mutations are easily detected and there are possibilities with some of the systems to detect new mutations. Due to increased demand there is now a number of commercially available tests developed, especially for the Applied Biosystems Sequence Detection Systems (ABI 7700 and 7000) and the LightCycler.
In this chapter some of the probe formats will be critically discussed in more detail, as well as exploring some examples where mutation detection has been widely used.

**Hybridisation Probes**

Mutation detection using hybridisation probes can be performed on the LightCycler system (Roche Molecular Diagnostics) using fluorescent resonance energy transfer (FRET) for detection (Wittwer et al., 1997). In the reaction two probes are used and FRET occurs when the donor probe is excited photometrically and transfers it energy to the acceptor probe causing the acceptor probe to emit light at a longer wavelength, which is measured by the instrument. The energy transfer can only occur when the two probes have hybridised to the target and are in close proximity.

Hybridisation probes can be used for mutation detection by performing a melting curve analysis directly after completing the amplification cycles. As the temperature is increased, the probes ‘melt’ or dissociate from the target at specific temperatures. The melting temperature (Tm) is the temperature at which 50% of the probe is dissociated from the template and this is observed as a decrease in fluorescent signal. When a mutation occurs in the target sequence the probe melts at a lower temperature than that of a perfect match (Lohmann et al., 2000). The melting temperatures are displayed as melting peaks by the LightCycler software and this allows wild-type, mutant and heterozygous genotypes to be distinguished (Lay and Wittwer 1997). Figure 1 shows an example of a typical melting curve.

**Probe Design**

The detection probe covers the mutation of interest and is usually 15-30 bases in length. The anchor probe requires a higher Tm than the detection probe or primers, typically 5-10°C higher. This will cause the detection probe to melt first and consequently the decrease in fluorescence is due to the detection probe, as a result the presence or
The absence of the mutation will be observed. The melting of the anchor probe is not detected. The higher Tm of this probe can be due to G-C content or probe length and as for any hybridisation probe the optimum spacing between the two probes is 1-3 bases. A useful tool for accurately predicting the melting temperature of the hybridisation probe is the Tm tool software available from Idaho Technology (http://www.idahotec.com) (Bernard et al., 1998).

The detection probes can be designed to either the mutant or wild-type sequence and to either the sense or anti-sense strand. Consideration should be given to the probe sequence that causes the most destabilised hybrids if the target contains a mismatch, in other words the hybrid containing the mutation will have a much lower Tm than the perfect match. Each mismatch has a different effect on the stability, for example, G-C mismatches are the most stable and consequently result in the smallest Tm shift, whereas T-C mismatches are the least stable and cause the largest Tm shift (Peyret et al., 1999). However, even the most stable mismatches can adequately separate mutants from wild-type (Bernard et al., 1998; Blomeke and Shields 1999).
PCR Conditions

The melting curve analysis is performed immediately after amplification by briefly denaturing the PCR product followed by cooling to 5-10°C below the Tm of the detection probe. The temperature is then slowly raised at a transition rate of 0.1-0.2°C per second whilst continuously monitoring fluorescence. As the probe melts the decrease in fluorescent signal is observed as a melting curve which is subsequently converted to a melting peak by the LightCycler software using the negative derivative of the melting curve. The melting peaks are easier to visualise and allow accurate determination of the Tm. To achieve good melting curves it is sometimes necessary to use asymmetric PCR to increase the amount of the target strand specific for the probe (Lyon et al., 1998; Phillips et al., 2000). This improves the melting curve because increased amplification of the target strand can reduce the competition between the template strands and template-probe hybridisations.

It is possible to detect more than one mutation during a reaction either by being able to easily resolve different melting peaks by Tm or by using different colour dyes for detection. Multiplexing by Tm uses the same acceptor probe with two different detector probes. The detector probes can be designed with different Tms by increasing the length of one or by differences in G-C content. It is possible to differentiate four melting peaks i.e. a perfect match and a mismatch for two mutations in this way (Bernard et al., 1998). Alternatively there are two detection channels on the LightCycler, which can be used to detect LC Red 640 and LC Red 705. Due to spectral overlap between the channels on the LightCycler it is necessary to correct for any cross talk between the dyes by running a colour compensation experiment on the machine. This calibration run monitors the emission from the different dyes over a temperature gradient and can then be applied to subsequent runs to remove the cross talk. Multiplexing the reactions significantly increases the information that can be obtained whilst minimising time and reagent costs. As for any multiplex, changes to cycling conditions and reaction conditions especially primer concentration and magnesium concentration may need to be made in order for all reactions to run optimally.
Advantages/Disadvantages

Hybridisation probes and melting curve analysis on the LightCycler offer a very rapid system for detection of mutations. A mutation detection assay can be completed in as little as 30 minutes, with the melting curve analysis requiring only five minutes. The system is semi-automated and requires no post-PCR handling, reducing the risk of PCR contamination. The results are easy to interpret and it is possible to detect previously unknown mutations provided they occur within the probe-binding site. Optimal probes are relatively simple to design. Standard primer design software can be used to calculate the binding efficiency and Tm, with flexibility in which DNA strand is targeted in order to design an optimal probe. The disadvantages of this system are the potential for multiplexing and the number of samples, which can be processed. The reactions can be multiplexed by Tm, which is dependent on designing probes with sufficiently different Tms that will be resolved by the software. In practice the melting peaks do not always discriminate as expected. The reactions can also be multiplexed by fluorophore, at present on the LightCycler only two dyes can be used although this has been increased to four with the release of the version 2.0 instrument. On the LightCycler the number of samples is limited to 32 although the PCRs can be performed quickly, so there is potential to perform multiple runs. Hybridisation probes can also be run on other platforms including the iCycler (BioRad) and the Rotor-Gene (Corbett Research).

ResonSense Probes

ResonSense probes are similar to hybridisation probes except that only a single probe is used (Lee et al., 2002). The probe is labelled with a fluorescent dye and FRET occurs between the probe and an intercalating dye, such as SYBR Green I present in solution. As with hybridisation probes ResonSense probes can be used for mutation detection by performing melting curve analysis (Gibson et al., 1999; Logan et al., 2001; Edwards et al., 2001). For design the same considerations as for hybridisation probes are required. As with hybridisation probes the probe requires to be blocked at the 3' end to
prevent it from acting as a primer, and this can be achieved either by placing the fluorescent label at the 3' end or by adding a blocker such as biotin or phosphate.

These probes offer an advantage over conventional hybridisation probes where sequence constraints prevent design of two probes. It may also be simpler to optimise the reactions with a single probe (Lee et al., 2002).

**Hydrolysis Probes**

An alternative hybridisation technique uses hydrolysis probes to detect PCR products in real-time (Livak et al., 1995). Hydrolysis probes are labelled at the 5' end with a fluorescent reporter molecule and at the 3' end with a quencher molecule. During extension the 5'→3' exonuclease activity of Taq polymerase cleaves the probe, emitting signal due to the separation of the reporter from the quencher. For mutation detection two probes are required, one targeting the wild-type sequence and the other targeting the mismatched sequence. These probes are labelled with different fluorescent reporter molecules to differentiate amplification of each of the sequences. Mismatches between the probe and the target reduce the efficiency of probe hybridisation and furthermore Taq polymerase is more likely to displace a mismatched probe rather than cleaving it, with no release of the reporter dye.

**Design of Hydrolysis Probes**

Standard design features that apply to other chemistries also apply to hydrolysis probes including length of 20–40 nucleotides, a G-C content of 40-60%, no runs of a particular nucleotide especially G, no repeated sequence motifs and a Tm of at least 5°C higher than the primers to ensure the probe has bound to the template before extension of the primers can occur (Landt, 2001). Two reporter dyes can be used to label hydrolysis probes for mutation detection, namely VIC and FAM and typically these probes are quenched by TAMRA. Mismatches
near the end of probes are not disruptive to hybridisation so it is recommended that the mutation is near the centre of the probe (Livak et al., 1995, 1995). The 5' end of the probe cannot be a guanosine residue, as a guanosine adjacent to the reporter dye will quench the reporter fluorescence even after probe cleavage (Livak et al., 1995). The estimated Tm for the probes should be between 65-67°C and it is recommended that the dedicated Primer Express software (Applied Biosystems) is used for the design.

**Minor-Groove Binding Probes**

A recent development based on hydrolysis probes is minor-groove binding probes (MGB). In this system the TAMRA quencher dye is replaced with a non-fluorescent quencher and a minor-groove binder stabilises the probe/target molecule by folding into the minor groove of the dsDNA (Kutyavin et al., 1997 and 2000). The increased stability and the increased Tm, due to the MGB, allows very short probes to be designed, typically 14 bp which is useful when sequence constraints limit the design of the probe. Additionally, because a non-fluorescent quencher is used the entire fluorescent signal observed is due to the reporter molecule which increases the accuracy of mutation detection. Software to aid with the design of MGB probes is available from Amersham Biosciences.

**Advantages/Disadvantages**

It is not possible to perform melting curve analysis when using hydrolysis probes as the probes are destroyed during the PCR reaction, therefore differently labelled hydrolysis probes are used for mutation detection. On most of the platforms available it is possible to multiplex two of these probes allowing mismatched and wild-type sequence to be detected in one reaction. Hydrolysis probes were initially designed for use on the ABI Sequence Detection Systems, where two dyes can be multiplexed and 96-384 samples analysed in 2 hours. This allows a rapid and relatively inexpensive system to be used for large-scale screening. Hydrolysis probes can be run on most other real-time
platforms but cannot always be multiplexed. This system is dependent on the inefficiency of the probes to bind to mismatched sequences and the fact that Taq polymerase is more likely to displace rather than cleave a mismatched probe. It is therefore conceivable to believe that this system can lead to false results being generated.

**Molecular Beacons**

Molecular Beacons are hybridising probes with a stem and loop structure (Tygai et al., 1998). They are composed of sequence complementary to the target sequence that forms the loop, flanked by arm sequences that are complementary to each other and form the stem. At the end of one arm-sequence a fluorescent molecule is attached and to the end of the other arm sequence is a quenching molecule. When the molecular beacon is free in solution the stem formation keeps the two dyes in close proximity causing quenching of the fluorescent molecule. However, when the probe is bound to the target molecule the fluorescent molecule is no longer quenched leading to increased fluorescence, which is measured. Molecular beacons are ideally suited for mutation detection due to the presence of the hairpin stem which means these probes bind only to perfectly matched sequences. This significantly increases the specificity when compared to linear probes such as hydrolysis or hybridisation probes (Tygai et al., 1998). The donor and acceptor dyes are not required to have overlapping spectra because they are brought into close proximity due to the confirmation of the stem and loop structure. This increases the number of potential fluorophores that can be used and detection is only limited by the hardware being used for the real-time detection (Tygai et al., 1998). As with MGB probes the quencher used is a non-fluorescent quencher (DABCYL) and so all fluorescent signal is due to the specific signal. However, great attention must be given to the design of these probes because the function of them is dependent upon correct hybridisation of the stem.
Probe Design

For mutation detection the molecular beacons are designed to be complementary to the mismatched base. The loop region is designed first and contains between 18-25 bases that give a melting temperature slightly higher than the annealing temperature of the primers. This causes the probe-target hybrid to be stable during annealing when the signal detection takes place (Mhlanga and Malmberg 2001). The arm sequences are usually 5-7 nucleotides and are designed to allow the stem to dissociate 7-10°C above the detection temperature, which is usually the annealing temperature of Taq polymerase in PCR. The Tm of the loop structure can be calculated using most primer design software that use % GC rule whereas the Tm of the stem sequence is best calculated using a DNA folding programme which will also ensure the dominant folding pattern is the intended hairpin (Mhlanga and Malmberg 2001). The SNP should be as close as possible to the centre of the probe. A software package that designs molecular beacons as well as primers is available from Premier Biosoft (Palo Alto, CA). The primers used are recommended to be sited 20-30 bp 3' or 5' of the molecular beacon respectively. The maximum product length should be no more than 250 bp as the signal has been shown to diminish in larger amplicons possibly due to the molecular beacon being unable to invade the double strands during the PCR annealing step (Mhlanga and Malmberg 2001).

Advantages/Disadvantages

The specificity of probe binding has been demonstrated to improve considerably when a hairpin stem is used and this is particularly useful for SNP detection (Tyagi et al., 1998). When a mismatch is present the probe/target hybrids are considerably less stable and dissociate at temperatures much lower than perfectly matched sequences and this temperature difference is much greater than that observed with linear probes. This allows use of one common temperature to detect several mutations (Gisendorf et al., 1998). Another advantage of the hairpin stem is that it brings the label moieties close together, allowing efficient quenching. This means a larger range of fluorescent dyes
can be employed offering greater flexibility for multiplexing. The main drawback of molecular beacons is the complex design ensuring that a hairpin structure will be formed. As with other probe systems molecular beacons are ideally suited to medium-scale semi-automated mutation detection.

**Scorpion Primers**

The previously described systems all require a fluorescent-labelled probe or probes for detection whereas the scorpion system does not (Thelwell et al., 2000). Like molecular beacons scorpion primers also take advantage of the stem-loop configuration. They consist of a hairpin linked to the 5' end of a PCR primer via a “PCR stopper” that prevents read-through by Taq polymerase and a loop, complementary to the target sequence. Attached to the 5' end of the stem is a donor fluorophore and positioned 3' of the donor just after the loop sequence is a quencher molecule (normally methyl red), so that when the stem forms the donor and quencher are opposite. When added to the reaction the scorpion primer is incorporated into the PCR product and during the cooling the scorpion primer folds back and hybridises to the complementary sequence in the newly synthesised part of the same DNA. This eliminates quenching and an increase in fluorescence is observed (Thelwell et al., 2000). The hybridisation reaction occurs within the same strand and hence occurs faster than hybridisation probes or hydrolysis probes (Didenko, 2001). Scorpion primers can be used for mutation detection by monitoring fluorescence at a temperature where the probe has dissociated from a target with a mismatch but remains bound to a complementary target (Thelwell et al., 2000).

Scorpions give a strong signal and act very quickly making them suitable for high throughput mutation detection assays. They are extremely versatile and, for example, are able to discriminate the most stable mismatches by decreasing the probe length until the mismatch becomes significant. This is not possible in other systems as shortening the probe length will most likely decrease the specificity whereas the probing mechanism used by scorpions increases the specificity as the
primer places the probe close to the desired binding site (Thelwell et al., 2000). Scorpion primers are suitable for use on most real-time platforms however the ability to multiplex is limited by the number of available detection channels.

**Advantages/Disadvantages**

The scorpion primer system utilises labelled primers that form a hairpin structure similar to molecular beacons and do not require an additional labelled probe(s) to be included. This has obvious cost advantages but also allows the reactions to be performed very quickly, as it is not necessary to wait for the probe to hybridise or for hydrolysis to occur (Thelwell et al., 2000). Additionally, there are no design constraints as with other systems on the position of the probe relative to the primers, which makes this an ideal system when there are sequence constraints. It is possible to multiplex scorpion primers and the only constraint is the number of dyes detectable by particular platforms. For example on the LightCycler only two dyes can be multiplexed however scorpions will also work on the ABI 7700 or the iCycler where up to four dyes can be used. The scorpion primers are ideally suited for a semi-automated system that is cost-effective and efficient at detecting multiple mutations.

**Fluorescent DNA Dyes**

SYBR Green I is a double-stranded specific DNA dye which can be used in real-time PCR for detection of product amplification and for detection of product Tm (Wittwer et al., 1997; Ririe et al., 1997). It has been used for detection of SNPs in products up to 200 bp by performing melting curve analysis (Lipsky et al., 2001; Elenitoba et al., 2001) although additional steps may have to be performed such as amplicon purification or addition of high concentration of SYBR Green I (Lipsky et al., 2001). Another intercalating dye which has been designed specifically for mutation detection is LCGreen I (Idaho technology) (Wittwer et al., 2003). Wittwer et al. (2003) have developed this dye and compared it to four other intercalating dyes.
including SYBR Green I and ethidium bromide and report that it is useful for detecting mutations without inhibiting or adversely affecting the PCR. Unlike fluorescent labelled probes, using fluorescent dyes can detect unknown mutations. When using a probe based system the probe requires to be designed specifically to the sequence of interest and although they can detect unknown mutations these must occur during the probe-binding site. Intercalating dyes can detect any mutation throughout the amplicon and can therefore be used for mutation screening with the added advantage of lower cost (Wittwer et al., 2003).

Applications of Real-Time PCR for Mutation Detection

As the ability to generate huge amounts of sequence data develops, information on sequence changes increases and this can be exploited for many purposes such as clinical diagnostics and microbiology. Molecular methods for SNP detection have been available for some time, however most of these methods are time consuming and cumbersome to perform. The development of real-time PCR has led to an explosion in cheap and rapid mutation detection methods. The applications in disease diagnostics for Factor V Leiden and Cystic Fibrosis will be explored, as well as the detection of antibiotic resistance mutations in bacteria.

Factor V Leiden

The first published assay for mutation detection was the detection of the Factor V Leiden mutation (Lay and Wittwer, 1997). It is a single point mutation in the factor V gene where a G is substituted for an A at position 1691, and it is a risk factor for venous thrombosis. Patients that are both homozygous and heterozygous for this mutation have an increased risk in developing the disease and, due to the high prevalence of the mutation there is an increased demand for clinical laboratories to provide fast, reliable and inexpensive tests.
The assay developed by Lay and Wittwer (1997) utilised a Cy5-labelled primer and a fluorescein labelled probe in a hybridisation system where the factor V Leiden mutation occurred 8 bases from the 5' end of the 23 bp probe. Amplification and melting curve analysis was performed on the LightCycler where after 45 rapid amplification cycles the PCR product was melted by slowly heating from 50°C to 75°C and fluorescence was continuously monitored. When wild-type sequence was detected the melting peak had a Tm of 66°C whereas a homozygous mutant resulted in a melting peak 8°C lower at 58°C. Heterozygous samples containing both sequences could also be detected and in these both melting peaks were observed. The assay was tested with 100 clinical samples and compared to the traditional method of PCR followed by restriction enzyme digestion and size separation. All 100 samples were correctly genotyped (Lay and Wittwer, 1997). The traditional method required at least 4-6 hours for completion whereas the LightCycler assay can be completed in just 30 minutes.

Assays have been developed for the ABI TaqMan using a TET-labelled and a FAM-labelled hydrolysis probe, one designed to match the wild-type sequence and the other to the mutant sequence (Sevall 2000) and for the Rotor-Gene using hybridisation probes (Ameziane et al., 2003). The latter two assays offer a higher throughput capacity than the LightCycler. A multiplex assay has also been developed to detect the factor V Leiden mutation as well as another mutation in the prothrombin gene that is associated with hereditary thrombophilia using hybridisation probes on the LightCycler (van den Bergh et al., 2000).

**Cystic Fibrosis**

Cystic Fibrosis (CF) is a common autosomal recessive disorder with a frequency of 0.05% and a carrier frequency of 5% (Rommens et al., 1989). It is caused by mutations in a gene on chromosome 7 (ABCC7) that produces the cystic fibrosis transmembrane conductance regulator protein. The transport of chloride ions across the membrane of epithelial cells is affected by the mutations and leads to a number of clinical symptoms. The most common mutation is a 3 bp deletion at codon 508.
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(F508del) and half of CF sufferers are homozygous for this mutation. A hybridisation probe assay was developed for the LightCycler that allows detection of this mutation in less than 30 minutes (Gundry et al., 1999). Two fluorescein-labelled hybridisation probes were designed to match the wild-type sequence perfectly from either codon 502 to 513 or from codon 504 to codon 511. The difference in the Tm between the wild-type and sequence containing the 3 bp deletion was approximately 10°C. During detection of this F508del mutation a second mutation in the same codon was detected. The temperature difference of the second mutation (F508C) was only 5°C from the wild-type and this allowed both mutations to be detected (Gundry et al., 1999). The second mutation was only observed because melting curve analysis was used and because the second mutation also occurred within the probe binding site. It would not have been detected by some methods, for example hydrolysis probes and demonstrates the potential power of melting curve analysis for mutation detection. In this case detection of the second mutation is particularly useful, as it is essential to test for this mutation to rule out false positives.

In a more recent study, scorpion primers have been used to detect five common CF-related mutations (Thelwell et al., 2000). In this system primers were designed close to the mutation site and in all but one the scorpion probe sequence was attached to the primer closest to the mutation site. The scorpion system was successfully used to distinguish wild-type and mutant sequence by having a probe that detects each type and which were run in separate reactions. Heterozygous samples were shown to have approximately half the level of fluorescence of wild-type sequence (Thelwell et al., 2000). Depending on the real-time platform used it is possible to multiplex the scorpion primers to provide a quick and inexpensive method for detection.

Detection of Antibiotic Resistance Mutations in Bacteria

Since 1999 there has been a number of studies performed detecting drug resistance in bacteria. These drug resistances are nearly always due to multiple mutations. These are most easily detected using melting curve
analysis on the LightCycler because hybridisation probes/ResonSense probes will bind to mismatched as well as perfectly matched sequences and therefore discriminate both the mutation they were designed to detect and any additional mutations which occur in the same binding site. In this way extra information can be obtained resulting in assays that detect multiple mutations whilst retaining the other benefits of the system including speed and simplicity. Table 1 summarises some of the organisms to which the LightCycler/hybridisation probe system has been applied. Detecting drug resistance mutations in this way has led to faster more reliable methods supplying information that is increasingly important as organisms develop more and more drug resistances. Two organisms which are slow or difficult to grow, will be explored in more detail.

**Mycobacteria tuberculosis**

*Mycobacterium tuberculosis* is responsible for three million deaths a year worldwide (Raviglione et al., 1995). Recommended treatment for tuberculosis (TB) comprises a combination of four drugs: rifampicin, isoniazid, pyrazinamide and ethambutol with or without streptomycin. Resistance has emerged to all of these drugs and multidrug resistance (MDR) in which the isolate is resistant to rifampicin and to at least one other drug is becoming more common (Brown et al., 2000). Rapid identification of drug resistance is necessary to minimise transmission of drug-resistant strains and to allow alternative drug therapy to be started (Edlin et al., 1992; Espinall et al., 2001). Rifampicin resistance
is well characterised and 95% of resistant *M. tuberculosis* strains have a mutation within an 81-bp region of the *rpoB* gene which encodes the β-subunit of the RNA polymerase (Kapur et al., 1994). Resistance to isoniazid is more complex with at mutations in at least four genes having been reported, though the highest proportion of mutations is in the *katG* gene.

In one study two changes in the *rpoB* gene at codon 531 and 526 and the most common mutation in the *katG* gene was detected using hybridisation probes on the LightCycler (Torres et al., 2000). The detector probe was designed to cover the mutation site. Using the *rpoB* probe the wild-type sequence gave a melting peak of 64°C, whereas a 2°C increase was detected when strains had a mutation at codon 531 and when strains had a mutation at codon 526 the melting peak was observed at 58°C *i.e.* a difference of 6°C. Using the *katG* probe, the wild-type strains melted at 72°C and two different mutations at codon 531 were detected, one melting 3°C lower and the other melting 5°C lower (Torres et al., 2000). This assay allowed detection of four of the most common drug-resistance related mutations using just two hybridisation probe pairs.

In a similar study, ResonSense probes (referred to as biprobes in the publication) were used to detect four rifampicin resistance mutations (Edwards et al., 2001). Three ResonSense probes were designed to cover three of the mutations and were labelled with Cy5. FRET occurred between the SYBR Green I, which was added to the PCR and between the Cy5-labelled probe and melting peaks were observed. The first probe spanned a mutation at codon 511 and resulted in a 4°C decrease in Tm when the mutation was present. The second probe spanned the mutation at 516 and resulted in a 7°C decrease in probe Tm. The final probe spanned a mutation at codon 526 and resulted in three melting peaks. The wild-type sequence melted at 61°C, whereas the mutation at codon 526 resulted in a melting temperature of 49°C and a mutation at codon 531, a mutation not spanned by the probe, resulted in a melting temperature of 66°C. It was not expected that the mutation at 531 would be detected however, it is thought that this sequence locus has the potential to form a relatively stable hairpin structure when in single-stranded form. It is believed that this structure destabilises
the probe/amplicon duplex and in strains with the 531 mutation, the single-stranded structures are less able to compete with the duplex structure. This explains why a higher melting temperature was observed compared to wild-type and was reproducibly demonstrated in 27 strains tested (Edwards et al., 2001). By using three ResonSense probes which all worked under the same cycling conditions, 98% of the mutations that were responsible for rifampicin resistance were easily detected. This study demonstrated that ResonSense probes are very flexible and can be used to detect mutations additional to those they were designed to detect but they can be affected by secondary structure and may not always perform as expected.

In a novel approach two hybridisation probe pairs were used to detect mutations in the rpoB gene but the probes were not used in the standard anchor-sensor design (Garcia de Viedema et al., 2002). Instead two adjacent pairs of FRET probes were designed to cover the entire 81 bp region of the rpoB gene. One pair of probes that were specific for the 5' half of the region and were labelled with fluorescein and LC Red 640. The other pair were specific for the 3' region and were labelled with fluorescein and LC Red 705. This allowed the probes to be multiplexed on the LightCycler so that a single-tube approach could be used. Using this method 12 different melting peaks were observed relating to 11 different mutations within the rpoB gene. In this way it is possible to search for a wider number of mutations and is particularly useful for detecting emerging mutations (Garcia de Viedema et al., 2002). The only potential drawback is that some of the detectable mutations may not relate to resistance.

**Helicobacter pylori**

*Helicobacter pylori* is a major cause of gastritis, peptic ulcer disease and gastric cancer (Blaser, 1997). Eradication using antibiotic therapy is recommended and one of the most widely used drugs is clarithromycin. However, resistance to clarithromycin is on the increase and is leading to treatment failure. Clarithromycin resistance is associated with three mutations in the peptidyltransferase encoding region of the 23S rRNA gene (Versalovic et al., 1996; Occhialini et al., 1997; Stone et al., 1997).
The first assay developed to detect these mutations utilised a single ResonSense probe labelled with Cy5 and was used on the LightCycler (Gibson et al., 1999). Clarithromycin sensitive strains had a probe melting peak at 68°C. Detection of two different mutations resulted in melting peaks of 63°C or 58°C. This method was originally designed for use on pure culture of the organism but has subsequently been applied to biopsy samples (Chisholm et al., 2001). This significantly increases the speed of diagnosis of drug resistance and allows appropriate therapy to commence.

Conclusions

As a result of whole genome sequencing projects there has evolved a need to be able to accurately detect mutations in a cost-effective, rapid and simple approach. Real-time PCR is suited for this approach and a number of different systems, both platforms and chemistries, have been evaluated for this purpose. Traditional methods that were used included restriction enzyme digestion, sequencing, single-stranded conformation polymorphism (SSCP) and specific PCR followed by direct hybridisation. Most of these methods are time consuming, slow and can often be expensive to perform especially when a large number of mutations are to be analysed.

Other new technologies have been applied to mutation detection and some may offer advantages over real-time PCR. Microarrays have been applied to mutation detection and allow numerous sequences to be analysed in parallel (Tillib and Mirzabekov 2001). Microarrays consist of amplifying PCR products that are either attached to the surface of a slide and are interrogated with specific probes or alternatively the specific probes are attached to the slide and the PCR products hybridised to them. In this way mutations can be detected in a large-scale format. The major advantage of this method is the ability to scan for large numbers of mutations in one experiment. However, at present it is difficult to distinguish all polymorphisms and difficult to detect low-level changes (Kirk et al., 2002). Microarrays are still dependent on performing PCR amplification and production of the array can be a very costly procedure. It is highly likely however, as microarray
technology improves and costs are reduced that this technology will be widely used for mutation detection especially when large-scale screening is required.

Sequencing has always had a role in mutation detection because of its accuracy and robustness although it is a time consuming and expensive method. However, pyrosequencing is ideally suited for SNP detection (Alderborn et al., 2000). Pyrosequencing is a novel sequencing method that measures the amount of pyrophosphate released as a result of nucleotide incorporation onto an amplified target (Ronaghi, 2003). It is a highly automated system that results in short sequence reads within 10-20 minutes for 96 samples. It is ideally suited for mutation detection where the base changes are sited close together. At present it is more expensive than using real-time PCR but it is finding an increasing role in mutation detection.

Real-time PCR offers a method for mutation detection that is extremely flexible, a large number of different approaches can be applied depending on the target of interest. The methods are all semi-automated and offer low to medium throughput depending on the platform used. The methods are very accurate in identifying the mutation and all platforms offer a close-tubed system that minimises cross-contamination in the laboratory. Additionally, the methods are relatively inexpensive and are easy to perform with simple analysis of results.

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