Real-Time PCR

Stephen A. Bustin
University of London, London, U.K.

INTRODUCTION

The real-time polymerase chain reaction (PCR) uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. This combines the DNA amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. Appropriate data analysis and/or use of apposite chemistries also eliminates the need for Southern blotting or DNA sequencing for amplicon identification. Its simplicity, specificity, and sensitivity, together with its potential for high throughput and the ongoing introduction of new chemistries, more reliable instrumentation, and improved protocols, has made real-time PCR the benchmark technology for the detection of DNA.

THE ASSAY

The concepts underlying fluorescence-based real-time PCR are straightforward and are described in detail in the accompanying review on real-time reverse transcription-PCR. Real-time PCR technology is based on the detection of a fluorescent signal produced proportionally during the amplification of a DNA target (Fig. 1). Rather than having to look at the amount of DNA target accumulated after a fixed number of cycles, real-time assays determine the point in time during cycling when amplification of a PCR product is first detected. This is determined by identifying the cycle number at which the reporter dye emission intensity rises above background noise. That cycle number is referred to as the threshold cycle (C_t). The C_t is determined at the exponential phase of the PCR reaction and is inversely proportional to the copy number of the target. Therefore the higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the lower the C_t. Real-time PCR assays are highly reproducible (Fig. 2A) and can easily discriminate between twofold differences in target numbers (Fig. 2B).

At its simplest, real-time PCR can be used as a qualitative assay. However, as fluorescence output is linear to sample concentration over a very broad range, this linear correlation between PCR product and fluorescence intensity can be used to calculate the amount of template present at the beginning of the reaction.

There has been an explosion of protocols, instruments, and chemistries, which on the one hand is evidence for the popularity and ubiquity of the assay, but also highlights the need to be aware of problems associated with the use of nonstandardized assays for diagnostic assays.[1]

CHEMISTRIES

Real-time PCR can utilize general nonspecific DNA-binding fluorophores (e.g., SYBER Green I), fluorophore-labeled primers (e.g., LUX™), or sequence-specific probes (e.g., Scorpions™).

Nonspecific Chemistries

An important advantage of nonspecific chemistries is that the design and setup of the assays is straightforward and, except for the cost of the real-time PCR apparatus, material costs are low. This makes them particularly attractive for the analysis of single nucleotide polymorphisms (SNP), which have become the markers of choice for identifying the multiple genes associated with complex diseases such as cancer or diabetes. Biallelic polymorphisms can be detected by combining allele-specific amplification with the detection of SYBR Green I. Allele-specific amplification takes advantage of the relative inability of *Taq* polymerase to extend primers that are mismatched to their targets at the 3′ end. The assay is carried out in two separate tubes, each of which contains a primer pair specific to one or the other allelic SNP variant.[2] Although there will be amplification of the mismatched allele, this occurs much less efficiently than that of the matching allele, delaying amplification and resulting in a much higher C_t being recorded. The specificity of the assay can be improved by using hairpin primers for the allele-specific PCR, as they are better than linear ones at discriminating between closely related sequences.[3]

The use of dissociation curve analysis to identify different amplicons obviates the requirement for two

DOI: 10.1081/E-EDGP 120020684

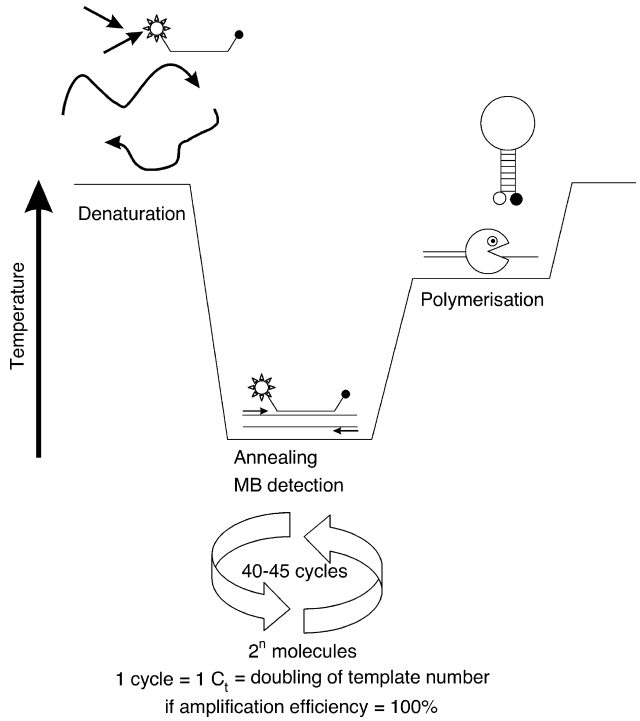

Fig. 1 Schematic representation of a real-time PCR assay using MB probes. At the annealing temperature, MB bind to their complementary target and fluorescence readings are taken. At the polymerization temperature MB dissociate from their target allowing *Taq* polymerase to read through and replicate the amplicon. A well-designed assay has an amplification efficiency of near 100% at the time fluorescence is first detected. Hence, any twofold difference in initial template concentration is reflected in a ΔC_t of 2.

separate amplification reactions. Following the PCR assay, double-stranded (ds) DNA product is melted into single-stranded (ss) DNA by a stepwise increase in temperature, with fluorescence data being collected at each temperature step. The magnitude of the reduction in fluorescence intensity of the SYBR Green dye due to its release from dsDNA provides an indicator of the amount of dsDNA dissociated at each step in the dissociation curve (Fig. 3). Furthermore, as different amplicons will melt at different temperatures, SYBR Green I can be used to distinguish different alleles through their melting temperatures (T_m). For example, Huntington's disease is caused by an expanded number of CAG repeats in the Huntington gene and the dissociation curve of a normal subject shows a single melting peak, whereas that of an affected individual has two.[4] Several closed-tube systems have been developed that can be used in combination with melting analysis of PCR products to identify both heterozygous and homozygous sequence variants,[5] and it has even been possible to develop triplex assays that use SYBR Green I and dissociation curves to identify different gene targets in the same tube.[6]

Fluorophore-Labeled Primers

Despite their attraction, there are several problems associated with the use of nonspecific dyes. In particular, there is a broadening of the melting transition and T_m compression among genotypes, which can lead to ambiguous results. Furthermore, dsDNA-specific dyes may redistribute during melting causing the release of dye from low-melting heteroduplexes and redistribution to higher melting heteroduplexes. The use of labeled primers for melting analysis avoids these problems while retaining the advantage of not having to use specific probes for each assay. The use of one fluorophore-labeled and one unlabeled primer enables melting profiles of the amplicon to be obtained immediately after completion of the PCR reaction and results in distinct melting curve shapes for different alleles, including those that differ by only a single base.[7]

Another method makes use of the principle of fluorescence resonance energy transfer (FRET). This is a distance-dependent interaction between the excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule at distances up to 70–100 Å without emission of a photon. As a result, the emission of the reporter fluorophore is quenched. In this approach, one of the amplification primers has a reporter and a quencher moiety attached to a hairpin structure at its 5′ end (Fig. 4A). When in solution, fluorescence emission from the reporter is quenched. A fluorescent signal is generated only when the labeled oligonucleotides are incorporated into the ds amplification product.[8] Labeled primer synthesis has been simplified recently by using the same type of hairpin primer, but only a single reporter fluorophore whose fluorescence emission is self-quenched based on sequence context.[9] These primers, also known as LUX™, quench when free in solution, fluoresce weakly when denatured, and emit light strongly when incorporated into DNA (Fig. 4B). Differential fluorescence labeling of primers allows the use of allele-specific PCR in a single tube.

Target-Specific Probes

Despite the evident usefulness of these chemistries, the specificity of these assays remains dependent on the specificity of the primers. Therefore the use of chemistries employing hybridization probes remains the reference method for genotyping. Two fluorogenic probes, labeled with two spectrally distinct dyes, are used to discriminate between the wild-type and mutant alleles. If amplification

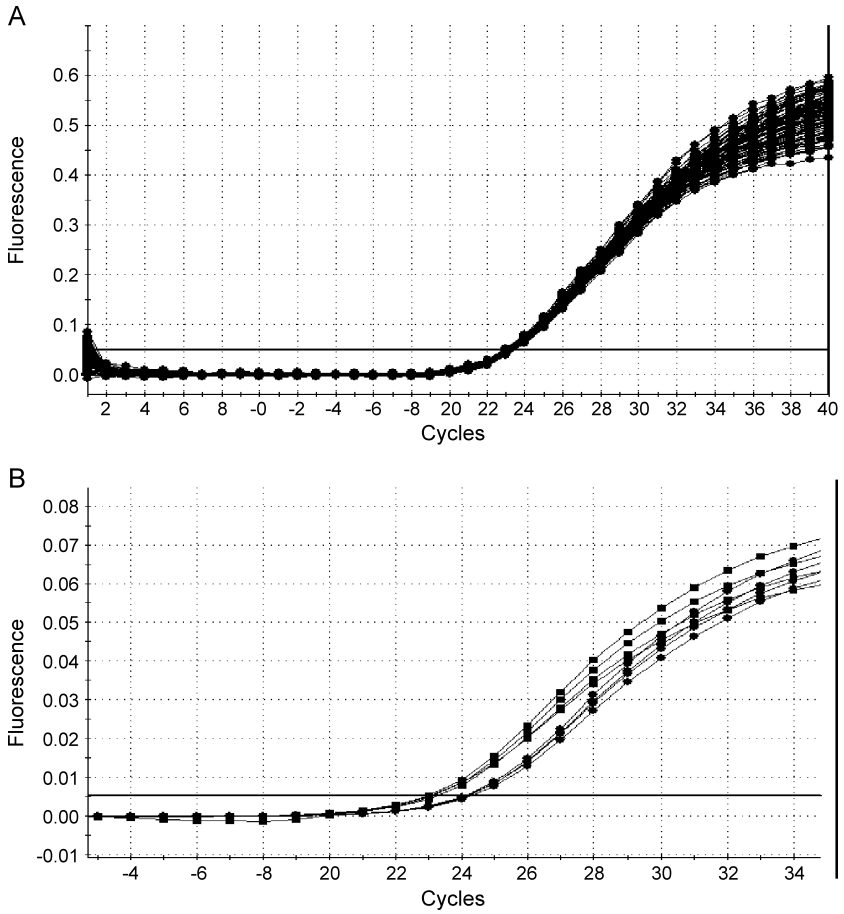

Fig. 2 Reproducibility and accuracy of real-time PCR. (A) Analysis of a 96-well plate containing replicates of a single template/mastermix. The assay was carried out on a Stratagene MX 4000 instrument using TaqMan™ chemistry. The average C_t for all 96 reactions is 23 ± 0.3. (B) Two reaction mixes were set up in triplicate. One contained 1×10^3 copies of template DNA and recorded a C_t of 23.1 ± 0.15. The other contained 2×10^3 copies and recorded a C_t of 24.1 ± 0.1. This corresponds exactly to the expected ΔC_t of 1.

in an unknown DNA sample is detected for the fluorophore identifying the wild-type allele but not for the one identifying the mutant allele, the sample is designated as wild-type homozygous. If amplification in an unknown DNA sample is detected for the fluorophore identifying the mutant allele but not for the dye identifying the wild-type allele, the sample can be designated as mutant homozygous. If the sample generates intermediate values for both dyes, it is designated as heterozygous for the two alleles (Fig. 5).

Assays based on the 5′-nuclease (‘‘TaqMan’’) use two allele-specific oligonucleotides that are labeled with different fluorophores at their 5′-ends. During PCR, fluorescence is generated after cleavage of the annealed probes by the 5′ nuclease activity of the Taq polymerase. Different sequences can de distinguished from one another by the differential fluorescence emission of the two reported dyes.[10]

Another assay, most commonly used on Roche's LightCycler™, uses two sequence-specific probes that bind adjacent to each other on the amplicon in a head-to-tail arrangement. One has a donor dye at its 3′-end, and the other has an acceptor dye on its 5′-end and is blocked at its 3′-end to prevent its extension during the annealing step. In solution, the two dyes are apart and only background fluorescence is emitted by the donor. Following the denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. The reporter is excited and passes its energy to the acceptor dye through FRET. For SNP/mutation detection one probe is positioned over the polymorphic site and the mismatch causes the probe to dissociate at a different temperature to the fully complementary amplicons. Melt curve analysis after the PCR reveals which alleles are present as one probe dissociating from the amplicon causes a decrease in fluorescence as FRET can no longer occur.[11]

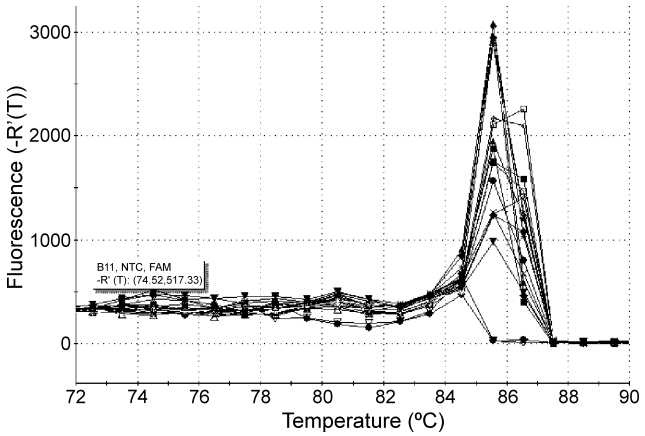

Fig. 3 Analysis of SYBR Green 1 dissociation curve. The first derivative view with respect to temperature provides a clear view of the rate of SYBR Green 1 loss and the temperature range over which this occurs. The example shows a view of data between 72°C and 90°C. The small peak at 74.5°C is probably due to primer dimer product formation as this is the only peak to occur in the NTC sample. The main peaks occur around 85.55°C although there are some with a distinctly different profile and a peak at 86.55°C. These distinct profiles represent different products in the final PCR product.

Molecular beacons (MB) and Scorpions are based on stem-loop hairpin structures, and MB in particular have found wide-ranging application as diagnostic tools.[12] Molecular beacons consist of a hairpin loop structure, with the loop complementary to a target nucleic acid and the stem formed by the annealing of complementary termini (Fig. 6A). One end of the stem has a reporter fluorophore attached and the other a quencher. In solution, free MB adopt a hairpin structure and the stem keeps the arms in close proximity, resulting in efficient proximal quenching of the fluorophore. During the denaturation step, the MB assume a random-coil configuration and fluoresce. At the annealing temperature, MB bind to any target amplicons as the probe/target duplex is designed to be thermodynamically more stable than the hairpin structure at that temperature. Once the probe binds to its target the hairpin is opened out and the fluorophore and quencher are separated, resulting in fluorescence. A major strength of this technology is the high specificity of the MB in recognizing nucleotide sequence mismatches in DNA and RNA. The hairpin shape of the MB causes mismatched probe/target hybrids to easily dissociate at significantly lower temperatures than exactly complementary hybrids. This is because the thermodynamic properties of the MB favor the formation of a hairpin form rather than continued hybridization to a less than perfectly matched target sequence. When the temperature is raised to allow primer extension, the MB dissociate from their targets and do not interfere with polymerization. A new hybridization takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplicon. Again, as this is a reversible process, melt curves can be used to analyze the dynamics of the reaction and determine the best temperature for fluorescent acquisition (Fig. 6B). There is some evidence to suggest that the measured signal ratios

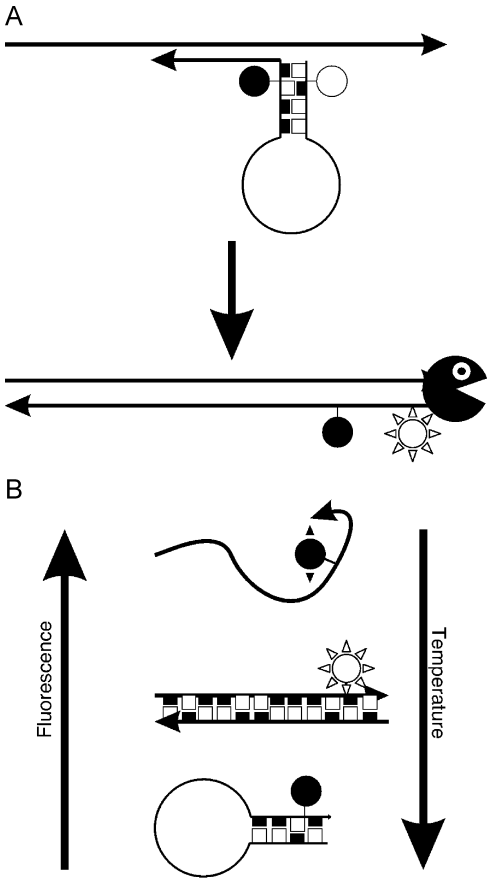

Fig. 4 Fluorophore-labeled probes. (A) The original design used a primer with a hairpin structure at its 5′-end that contained a fluorophore and quencher at opposite ends of the hairpin. During the first cycle of PCR the primers are extended and become templates during each subsequent cycle. This linearizes the hairpin, separates the donor and acceptor moieties, and results in fluorescence emission from the fluorophore. (B) LUX™ primers. One primer contains a fluorophore, the other one is unlabeled. The fluorogenic primer has a short sequence tail of 4–6 nucleotides on the 5′-end that is complementary to the 3′-end of the primer. The resulting hairpin secondary structure provides optimal quenching of the attached fluorophore. When the primer is incorporated into the double-stranded PCR product, the fluorophore is dequenched and a signal is reported.

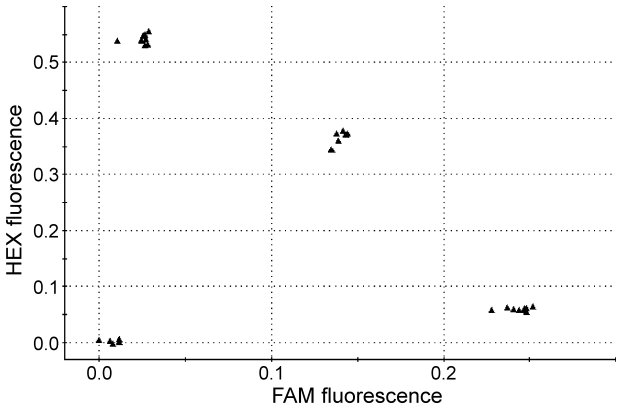

Fig. 5 Scatter plot for real-time SNP analysis using MB. Fluorescence is reported during each annealing step when the MB is bound to its complementary target and the C_t value for each dye in the sample is used to determine the genotype of the samples. A C_t value equal to the final cycle of the PCR reaction (typically 40 or 45) indicates the absence of a specific allele. Each plotted point represents the coordinates of the C_t values for the two dyes in a single well. Here the x axis corresponds to FAM C_t whereas the y axis corresponds to HEX C_t and the plotted points (x,y) correspond to the coordinates describing the two C_t values determined for a given well. The position of the data point for a given well on the scatter plot indicates the presence or absence of each allele, providing a rapid method for clustering the samples that are homozygous for either of the two alleles or are heterozygous. If a highly quantitative measurement of copy numbers is not necessary, it is also possible to measure fluorescence when cycling is complete.

with MB assays are proportional to the amount of the minor allele over a wider range than with the TaqMan assay.[13] Multiplexing of MB is enhanced by the use of wavelength-shifting MB (Fig. 6C).[14] These extend the range of fluorophore/quencher pairs that will function at a given wavelength and contain a generic harvester fluorophore, a probe-specific emitter fluorophore, and a quencher. Scorpions were originally made up of a single oligonucleotide containing a 5′ fluorophore, a stem-loop structure containing the probe, a quencher, and a PCR blocker to prevent read-through by DNA polymerase and primer. In the presence of a target, the specific probe sequence folds back on itself to bind its complement within the same DNA strand, opening up the hairpin loop and separating the fluorophore and quencher. This unwieldy structure was replaced by the second-generation Scorpions that are made up of two oligonucleotides. One contains the fluorophore-coupled probe, the other a quencher-coupled complementary sequence. For SNP analysis, the fluorescence is monitored above the T_m of the mismatch probe/target duplex and below the T_m of the fully complementary probe/target duplex. Under these conditions the mismatched probe reassociates with the quencher element to become nonfluorescent, whereas the hybridized wild-type probe is separated from the quencher element and is fluorescent. Because the hybridization of probe sequence to amplicon is intramolecular, Scorpion probes are more efficient than binary systems such as MB and as a result generate significantly greater signal intensities.[15]

There are many more probe chemistries available, all with their own advantages and disadvantages. These include Hybeacons, which require only a single fluorophore and make use of the quenching properties of DNA. This makes them easy to design and synthesize.[16] Light-up probes are composed of thiazole orange conjugated to peptide nucleic acid (PNA) (see below) and combine the excellent hybridization properties of PNA, which allows the use of shorter probes, with the extraordinary fluorescence enhancement of asymmetric cyanine dyes upon binding to nucleic acids.[17] Eclipse™ probes are linear probes that have a minor groove binder (MGB) and quencher on the 5′-end and the fluorophore on the 3′-end.[18] This is the other way round compared with hydrolysis probes, and the presence of the MGB at the 5′-end prevents cleavage of the Eclipse probe by *Taq* polymerase. Other chemistries are described in detail elsewhere.[19]

Real-time PCR assays generally use symmetric primers. However, this results in the reactions typically slowing down and entering the plateau phase in a stochastic manner, because reannealing of the template strands gradually outcompetes primer and probe binding to the template strands. This is a particular problem when the aim is to detect specific DNA targets down to alleles of single-copy genes in single cells. Asymmetric PCR potentially circumvents the problem of amplicon strand reannealing by using unequal primer concentrations. However, asymmetric amplification is much less efficient and requires extensive optimization to identify the proper primer ratios, the amounts of starting material, and the number of amplification cycles that can generate reasonable amounts of product for individual template/target combinations. A recent innovation termed linear-after-the-exponential-PCR (LATE-PCR) uses unequal primer concentrations but takes into account the effect of the actual primer concentrations on primer T_m.[20] It corrects for the fact that the T_m of the limiting primer is often several degrees below the T_m of excess primer and allows the asymmetric PCR to proceed as efficiently as symmetric PCR. Furthermore, ss amplicons are generated with predictable kinetics for many cycles beyond the exponential phase. This permits uncoupling of primer annealing from product detection. As a result, the T_m of the probe no

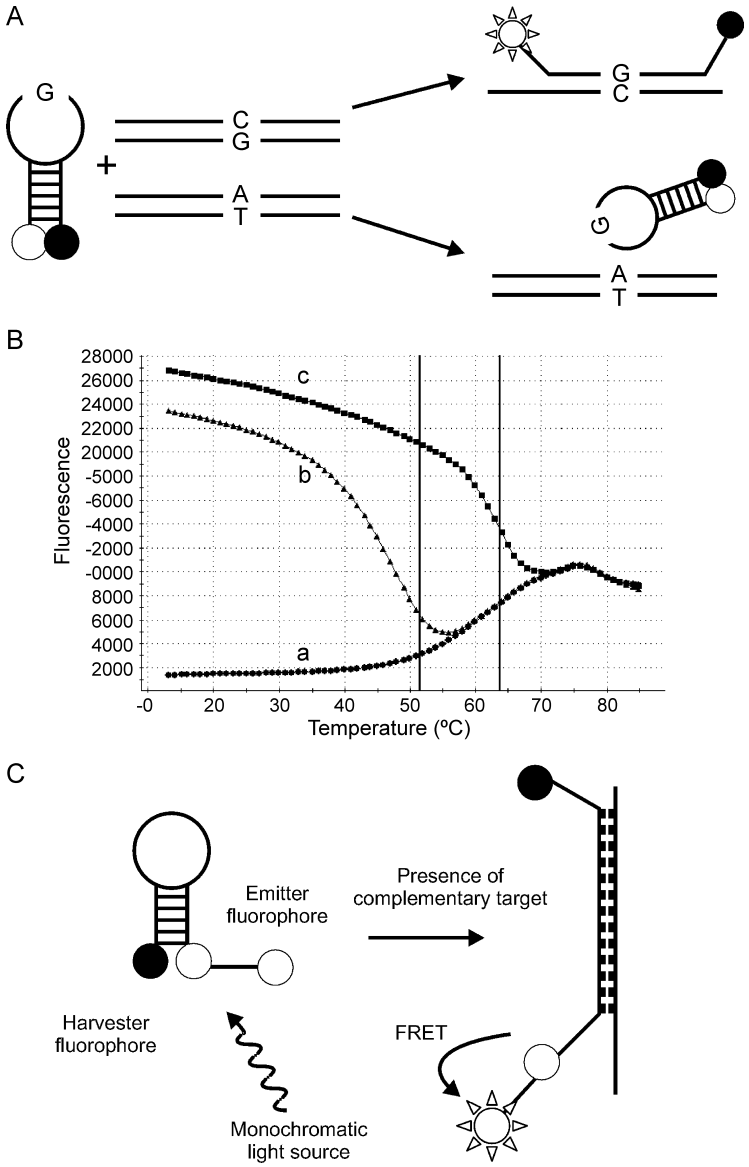

Fig. 6 Molecular beacons and melting curves. (A) Structured probes are better at discriminating single base-pair mismatches than linear ones such as TaqMan™. Only one MB is shown, which hybridizes to its perfect complement and emits fluorescence but remains closed and unbound to a target with a single nucleotide mismatch. (B) Molecular beacon melting profile for allelic discrimination. Three melting curves are visible. One for MB alone (a), a second one for MB and its perfectly complementary single-stranded oligonucleotide target (b), and a third one for MB plus a single-strand target that produces a probe/target hybrid containing a single mismatched base pair (c). The two vertical bars indicate the optimal annealing temperature range in which the perfectly matched MB will have greater fluorescence than the mismatched MB, with background fluorescence still low. (C) Wavelength shifting MB. The MB has two fluorophores on one end, a ''harvester'' and an ''emitter,'' and a quencher on the other end. In the hairpin loop structure, the quencher forms a nonfluorescent complex with the harvester. Upon hybridization of the MB to a complementary sequence, quenching of the harvester fluorophore is relieved, and it transfers energy via FRET to the emitter, which emits fluorescence.

longer needs to be higher than the T_m of either primer. This permits the use of low-T_m probes, which are inherently more allele-discriminating, generate lower background, and can be used at saturating concentrations without interfering with the efficiency of amplification.

Another important advantage of using probe-based chemistries is that it is possible to multiplex, i.e., amplify multiple targets in a single tube, as fluorescent dyes with different emission spectra may be attached to the different probes (Fig. 7). Probes afford a level of discrimination

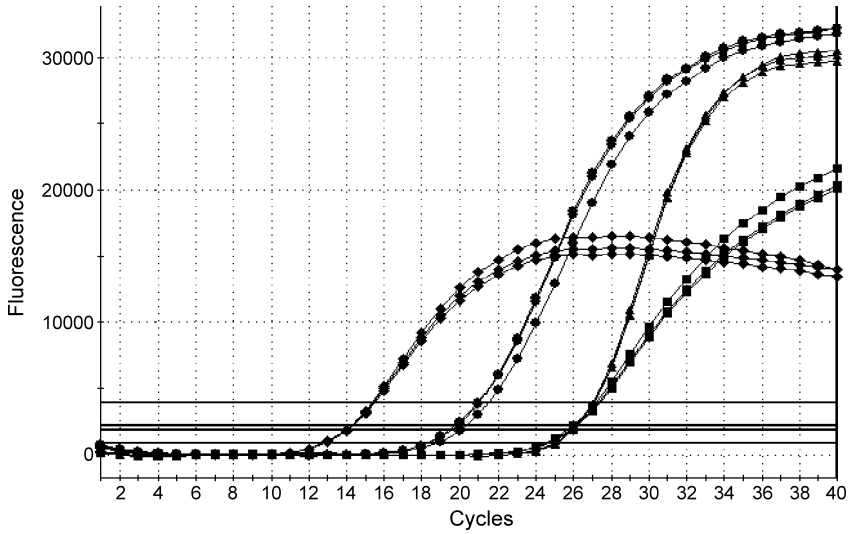

Fig. 7 Multiplex PCR. Four TaqMan™ probes were labeled with FAM, HEX, ROX, or Cy5, and a PCR assay was performed using the Stratagene ''Brilliant® Multiplex QPCR'' master mix on the MX4000 real-time PCR system. Each sample was analyzed in triplicate. The four horizontal lines indicate the four thresholds calculated for the individual fluorophores. The four targets differ by 14 C_ts, which translates into a 1.6×10^4-fold difference in target abundance.

impossible to obtain with SYBR Green, as they will only hybridize to true targets in a PCR and not to primer–dimers or other spurious products.

DNA ANALOGUES

A recent development has been the use of probes incorporating peptide nucleic acid (PNA) or locked nucleic acid (LNA™). In PNA the entire deoxyribose phosphate backbone is replaced by a structurally homomorphous backbone consisting of repeating *N*-(2-aminoethyl)-glycine units linked by peptide bonds. As the PNA backbone is not charged, hybridization is not affected by intrastrand repulsion, and no salt is necessary to facilitate and stabilize the formation of PNA and DNA or RNA duplexes. Furthermore, the ΔT_m of a single PNA/DNA mismatch is significantly higher than that of a DNA/DNA mismatch, making PNA probes useful for mismatch detection. PNA technology has been used in an ingenious approach utilizing quencher-labeled primers. This system uses two labeled molecules: 1) a quencher-labeled PNA probe with a C-terminal DABCYL group; and 2) a primer with a target-specific sequence at its 3′-end and a fluorophore and a PNA-probe-complementary sequence tag at its 5′-end.[21] The PNA/primer duplex has a T_m higher than the primer annealing temperature, but lower than the T_m of the primer/amplicon duplex. This ensures that excess primer is quenched at the annealing temperature and the fluorescence measured during the annealing step indicates the amount of primer hybridized to amplicon, plus any full-length ds amplicon as the end result is a fluorescently labeled ds amplicon. The main disadvantage with PNA probes is that they can aggregate and precipitate.

In LNA the furanose ring conformation is restricted by a methylene bridge that connects the 2′-oxygen position of ribose to the 4′-carbon. This bridge reduces the conformational flexibility of the ribose, which imparts superior affinity and specificity in binding complementary sequences of DNA or RNA. In addition, the change in T_m caused by a mismatch is significantly greater with a LNA/DNA duplex than with of a DNA/DNA duplex, resulting in enhanced specificity for SNP/mutation analyses.[22] Using appropriate dyes, it is possible to use probes as short as 7 nucleotides long for mismatch discrimination, making it possible to generate universal genotyping reagents. Unlike PNA, there is no problem with solubility of LNA molecules.

OTHER APPLICATIONS

The real-time PCR-based focus on pathogen genotype is having a significant impact on the detection of viruses, bacteria, and parasites in diagnostic microbiology.[23] The biggest application of real-time technology is probably in virology, where these assays have been used to investigate the role of viruses in a range of human diseases. The ability to multiplex has allowed the reliable measurement

of different viral nucleic acid targets within a single sample, and the discrimination of multiple viral genotypes within a single reaction tube. A major application is in the detection of viral load, and real-time assays have become very useful as indicators of the extent of active infection, host/virus interaction, and the efficacy of antiviral treatment. Other uses include the assessment of viral gene therapy vectors before their use in clinical trials and the study of new and emerging viruses and clinical symptoms experienced by patients.[24]

A major benefit lies in the rapidity with which results can be obtained. This is of major importance when detecting bacterial pathogens, as it allows a specific and timely application of antibiotics. Real-time assays are ideal for distinguishing between different serotypes of a single bacterial species,[25] for detecting and monitoring drug resistance among clinical isolates,[26] for detecting pathogens in food,[27] and not least for identifying microbes used as agents of biological warfare.[28]

The worldwide approval of a large number of genetically modified organisms (GMOs) among countries, and the associated labeling requirements, has resulted in the development of real-time PCR-based methods for the detection of the presence of GMO in food or food additives.[29] Genetically modified organism detection through PCR relies on parallel amplification of the transgene and of an endogenous reference gene that provides a control both for the lack of inhibition and for the ability to amplify the target DNA in the sample. Additionally, for quantitative analyses, amplification of the reference gene provides an estimation of the total amount of target DNA present in the sample. Targeting the DNA is particularly appropriate, because of the high stability of this molecule under the extreme conditions used during processing of some food products.

CONCLUSION

These examples are but a few of the huge number of applications that have benefited from the introduction of real-time fluorescence-based PCR assays and that have contributed to the transformation of this technique from an experimental tool into the scientific mainstream.[30] The advances in robotic nucleic acid extraction and liquid-handling systems, together with the continuous introduction of less expensive, yet more capable thermal cyclers make real-time PCR an attractive and essential technology for routine diagnostics. Recent developments in multiplexing make it possible to envisage easy identification, genotyping, and quantification of DNA targets in single, rapid reactions. However, the technology is only as reliable as the accompanying controls and associated quality-assurance programs. This includes the quality of standards, the use of suitably controlled standard curves, and the need to fully optimize, validate, and evaluate each new assay against previously standardized assays. Nevertheless, it is clear that real-time PCR is a technique whose time has come.[31]

ACKNOWLEDGMENTS

I am grateful to the charity Bowel and Cancer Research for financial support.

REFERENCES

1. Niesters, H.G. Molecular and diagnostic clinical virology in real time. Clin. Microbiol. Infect. **2004**, *10*, 5–11.
2. Germer, S.; Holland, M.J.; Higuchi, R. High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. Genome Res. **2000**, *10*, 258–266.
3. Hazbon, M.H.; Alland, D. Hairpin primers for simplified single-nucleotide polymorphism analysis of Mycobacterium tuberculosis and other organisms. J. Clin. Microbiol. **2004**, *42*, 1236–1242.
4. Elenitoba-Johnson, K.S.; Bohling, S.D.; Wittwer, C.T.; King, T.C. Multiplex PCR by multicolor fluorimetry and fluorescence melting curve analysis. Nat. Med. **2001**, *7*, 249–253.
5. Wittwer, C.T.; Reed, G.H.; Gundry, C.N.; Vandersteen, J.G.; Pryor, R.J. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin. Chem. **2003**, *49*, 853–860.
6. Hernandez, M.; Rodriguez-Lazaro, D.; Esteve, T.; Prat, S.; Pla, M. Development of melting temperature-based SYBR Green I polymerase chain reaction methods for multiplex genetically modified organism detection. Anal. Biochem. **2003**, *323*, 164–170.
7. Gundry, C.N.; Vandersteen, J.G.; Reed, G.H.; Pryor, R.J.; Chen, J.; Wittwer, C.T. Amplicon melting analysis with labeled primers: A closed-tube method for differentiating homozygotes and heterozygotes. Clin. Chem. **2003**, *49*, 396–406.
8. Nazarenko, I.A.; Bhatnagar, S.K.; Hohman, R.J. A closed tube format for amplification and detection of DNA based on energy transfer. Nucleic Acids Res. **1997**, *25*, 2516–2521.
9. Nazarenko, I.; Lowe, B.; Darfler, M.; Ikonomi, P.; Schuster, D.; Rashtchian, A. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucleic Acids Res. **2002**, *30*, e37.
10. Abbaszadegan, M.R.; Struewing, J.P.; Brown, K.M.; Snider, J.V.; Goodsaid, F.; Gore-Langton, R.; Hughes, M.R. Automated detection of prevalent mutations in BRCA1 and BRCA2 genes, using a fluorogenic PCR allelic discrimination assay. Genet. Test. **1997**, *1*, 171–180.
11. Aslanidis, C.; Nauck, M.; Schmitz, G. High-speed

prothrombin G→A 20210 and methylenetetrahydrofolate reductase C→T 677 mutation detection using real-time fluorescence PCR and melting curves. BioTechniques **1999**, *27*, 234–238.
12. Mhlanga, M.M.; Malmberg, L. Using molecular beacons to detect single-nucleotide polymorphisms with real-time PCR. Methods **2001**, *25*, 463–471.
13. Tapp, I.; Malmberg, L.; Rennel, E.; Wik, M.; Syvanen, A.C. Homogeneous scoring of single-nucleotide polymorphisms: Comparison of the 5′-nuclease TaqMan assay and molecular beacon probes. BioTechniques **2000**, *28*, 732–738.
14. Tyagi, S.; Marras, S.A.; Kramer, F.R. Wavelength-shifting molecular beacons. Nat. Med. **2000**, *18*, 1191–1196.
15. Solinas, A.; Brown, L.J.; McKeen, C.; Mellor, J.M.; Nicol, J.; Thelwell, N.; Brown, T. Duplex Scorpion primers in SNP analysis and FRET applications. Nucleic Acids Res. **2001**, *29*, E96.
16. French, D.J.; Archard, C.L.; Andersen, M.T.; McDowell, D.G. Ultra-rapid DNA analysis using HyBeacon probes and direct PCR amplification from saliva. Mol. Cell. Probes **2002**, *16*, 319–326.
17. Timcheva, I.I.; Maximova, V.A.; Deligeorgiev, T.G.; Gadjev, N.I.; Sabnis, R.W.; Ivanov, I.G. Fluorescence spectral characteristics of novel asymmetric monomethine cyanine dyes in nucleic acid solutions. FEBS Lett. **1997**, *405*, 141–144.
18. Afonina, I.A.; Reed, M.W.; Lusby, E.; Shishkina, I.G.; Belousov, Y.S. Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. BioTechniques **2002**, *32*, 940–949.
19. Bustin, S.A.; Nolan, T. Chemistries. In *A–Z of Quantitative PCR*; Bustin, S.A., Ed.; URL Press: La Jolla, CA, 2004.
20. Sanchez, J.A.; Pierce, K.E.; Rice, J.E.; Wangh, L.J. Linear-after-the-exponential (LATE)-PCR: An advanced method of asymmetric PCR and its uses in quantitative real-time analysis. Proc. Natl. Acad. Sci. U. S. A. **2004**, *101*, 1933–1938.
21. Fiandaca, M.J.; Hyldig-Nielsen, J.J.; Gildea, B.D.; Coull, J.M. Self-reporting PNA/DNA primers for PCR analysis. Genome Res. **2001**, *11*, 609–613.
22. Johnson, M.P.; Haupt, L.M.; Griffiths, L.R. Locked nucleic acid (LNA) single nucleotide polymorphism (SNP) genotype analysis and validation using real-time PCR. Nucleic Acids Res. **2004**, *32*, e55.
23. Mackay, I.M. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. **2004**, *10*, 190–212.
24. Mackay, I.M.; Arden, K.E.; Nitsche, A. Real-time PCR in virology. Nucleic Acids Res. **2002**, *30*, 1292–1305.
25. Probert, W.S.; Bystrom, S.L.; Khashe, S.; Schrader, K.N.; Wong, J.D. 5′ Exonuclease assay for detection of serogroup Y *Neisseria meningitidis*. J. Clin. Microbiol. **2002**, *40*, 4325–4328.
26. Esaki, H.; Noda, K.; Otsuki, N.; Kojima, A.; Asai, T.; Tamura, Y.; Takahashi, T. Rapid detection of quinolone-resistant *Salmonella* by real time SNP genotyping. J. Microbiol. Methods **2004**, *58*, 131–134.
27. McKillip, J.L.; Drake, M. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. J. Food Prot. **2004**, *67*, 823–832.
28. Qi, Y.; Patra, G.; Liang, X.; Williams, L.E.; Rose, S.; Redkar, R.J.; DelVecchio, V.G. Utilization of the rpoB gene as a specific chromosomal marker for real-time PCR detection of *Bacillus anthracis*. Appl. Environ. Microbiol. **2001**, *67*, 3720–3727.
29. Vaitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. J. Agric. Food Chem. **1999**, *47*, 5261–5266.
30. Ginzinger, D.G. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Exp. Hematol. **2002**, *30*, 503–512.
31. Walker, N.J. A technique whose time has come. Science **2002**, *296*, 557–559.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016