 10.1111/j.1198-743X.2004.00722.x

Real-time PCR in the microbiology laboratory

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ABSTRACT

Use of PCR in the field of molecular diagnostics has increased to the point where it is now accepted as the standard method for detecting nucleic acids from a number of sample and microbial types. However, conventional PCR was already an essential tool in the research laboratory. Real-time PCR has catalysed wider acceptance of PCR because it is more rapid, sensitive and reproducible, while the risk of carryover contamination is minimised. There is an increasing number of chemistries which are used to detect PCR products as they accumulate within a closed reaction vessel during real-time PCR. These include the non-specific DNA-binding fluorophores and the specific, fluorophore-labelled oligonucleotide probes, some of which will be discussed in detail. It is not only the technology that has changed with the introduction of real-time PCR. Accompanying changes have occurred in the traditional terminology of PCR, and these changes will be highlighted as they occur. Factors that have restricted the development of multiplex real-time PCR, as well as the role of real-time PCR in the quantitation and genotyping of the microbial causes of infectious disease, will also be discussed. Because the amplification hardware and the fluorogenic detection chemistries have evolved rapidly, this review aims to update the scientist on the current state of the art. Additionally, the advantages, limitations and general background of real-time PCR technology will be reviewed in the context of the microbiology laboratory.

Keywords Real-time PCR, quantitation, molecular, diagnostics

Accepted: 26 December 2002

Clin Microbiol Infect 2004; 10: 190–212

BACKGROUND

Diagnostic microbiology is in the midst of a new era. Rapid nucleic acid amplification and detection technologies are quickly displacing the traditional assays based on pathogen phenotype rather than genotype. The polymerase chain reaction (PCR) [1,2] has increasingly been described as the latest gold standard for detecting some microbes, but such claims can only be taken seriously when each newly described assay is suitably compared to its characterised predecessors [3–6]. PCR is the most commonly used nucleic acid amplification technique for the diagnosis of infectious disease, surpassing the probe and signal amplification methods. The PCR can amplify DNA or, when preceded by a reverse transcription (RT) incubation at 42–55 °C, RNA. RT-PCR is the most sensitive method for the detection and quantitation of mRNA, especially for low-abundance templates [7–10]. The PCR process can be divided into three steps. First, double-stranded DNA (dsDNA) is separated at temperatures above 90 °C. Second, oligonucleotide primers generally anneal at 50–60 °C, and, finally, optimal primer extension occurs at 70–78 °C. The temperature at which the primer anneals is usually referred to as the T_M. This is the temperature at which 50% of the oligonucleotide–target duplexes have formed. In the case of real-time PCR, the oligonucleotide could represent a primer or a labelled probe. The T_M differs from the denaturation temperature (T_D), which refers to the T_M as it applies to the melting of dsDNA. The rate

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of temperature change or ramp rate, the length of the incubation at each temperature and the number of times each cycle of temperatures is repeated are controlled by a programmable thermal cycler. Current technologies have significantly shortened the ramp rates, and therefore assay time, through the use of electronically controlled heating blocks or fan-forced heated air flows.

The traditional diagnostic microbiological assays include microscopy, microbial culture, antigenaemia and serology. These can be limited by poor sensitivity, slow-growing or poorly viable organisms, narrow detection windows, complex interpretation, immunosuppression, antimicrobial therapy, high levels of background and non-specific cross-reactions [11,12]. Nonetheless, microbial culture produces valuable epidemiological data, revealing new, uncharacterised or atypical microbes and yielding intact or infectious organisms for further study [13]. It is therefore clear that the role of the traditional assay continues to be an important one [14–18]. Additionally, PCR has some significant limitations. Our ability to design oligonucleotide primers only extends to our knowledge of a microorganism's genome as well as the ability of publicly available sequence databases to suitably represent all variants of that microbe. It is common for microbial genomes to contain unexpected mutations, which reduce or abrogate the function of a PCR. Traditionally, false-positives due to carryover contamination have caused considerable problems in the routine implementation of PCR in the diagnostic laboratory and have led to strict guidelines for the design of laboratories dedicated to performing PCR. Additionally, PCR may be too sensitive for some applications, detecting a microbe that is present at non-pathogenic levels. Thus, care is required when designing a PCR assay and interpreting its results.

Existing combinations of PCR and amplicon detection assays will be called 'conventional PCR' throughout this review. The detection components include agarose gel electrophoresis [19], Southern blot [20] and ELISA-like systems [21]. Conventional PCR has been used to obtain quantitative data, with promising results [22]. However, these approaches have suffered from the laborious post-PCR handling steps required to evaluate the amplicon [23].

The possibility that, in contrast to conventional PCR, the detection of amplicon could be visualised as the amplification progressed was a welcome one. This expanded the role of PCR from that of a pure research tool to that of a versatile technology permitting the development of routine diagnostic applications for the high- and low-throughput clinical microbiology laboratory [24,25]. Along the way, real-time assays have provided insight into the kinetics of the PCR as well as the efficiency of different nucleic acid extraction methods and the role that some compounds play in the inhibition of amplification [20,26–33]. Real-time PCR has made many more scientists familiar with the crucial factors contributing to successful amplification of nucleic acids. Today, real-time PCR is used to detect nucleic acids from food, vectors used in gene therapy protocols, genetically modified organisms, and areas of human and veterinary microbiology and oncology [34–36].

The monitoring of accumulating amplicon in real time has been made possible by the labelling of primers, oligonucleotide probes (oligoprobes) or amplicons with molecules capable of fluorescing. These labels produce a change in signal following direct interaction with, or hybridisation to, the amplicon. The signal is related to the amount of amplicon present during each cycle and will increase as the amount of specific amplicon increases. These chemistries have clear benefits over earlier radiogenic labels, including an absence of radioactive emissions, easy disposal and an extended shelf-life [37].

A significant improvement introduced by real-time PCR is the increased speed with which it can produce results. This is largely due to the reduced cycle times, removal of separate post-PCR detection procedures, and the use of sensitive fluorescence detection equipment, allowing earlier amplicon detection [38,39]. A reduced amplicon size may also play a role in this speed; however, it has been shown that decreased product size does not strictly correlate with improved PCR efficiency, and that the distance between the primers and the oligoprobe may play a more significant role [40,41].

The technical disadvantages of using real-time PCR instead of conventional PCR include the need to break the seal of an otherwise closed system in order to monitor amplicon size, the incompatibility of certain platforms with some fluorescent chemistries, and the relatively restricted multiplex capabilities of current systems. Additionally, the

start-up expense of real-time PCR may be prohibitive for low-throughput laboratories.

Because most of the popular real-time PCR chemistries involve hybridisation of an oligoprobe(s) to a complementary sequence on one of the amplicon strands, the inclusion of more of the primer that creates that strand is beneficial to the generation of an increased fluorescent signal [42]. We have found that this asymmetric PCR approach improves the signal from both our conventional and real-time oligoprobe-hybridisation assays.

Although some of the fluorescent labels have been given an associated nomenclature by their developer, the term 'fluorophore' will generally be used to describe these moieties, while their inclusion as labels on an oligonucleotide will be described as rendering it 'fluorogenic'. The most commonly used fluorogenic oligoprobes rely upon fluorescence resonance energy transfer (FRET) between fluorogenic labels or between one fluorophore and a dark or black-hole non-fluorescent quencher (NFQ), which disperses energy as heat rather than fluorescence [43]. FRET is a spectroscopic process by which energy is passed between molecules separated by 10–100 Å that have overlapping emission and absorption spectra [44–46]. Förster primarily developed the theory behind this process, which is a non-radiative induced dipole interaction [43,47,48].

As alluded to earlier, post-amplification manipulation of the amplicon is not required for real-time PCR, because the fluorescent signals are directly measured as they pass out of the reaction vessel, so real-time PCR is often described as a 'closed' or homogeneous system. Apart from the time saved by amplifying and detecting template in a single tube, there is minimal potential for carryover contamination, and the assay's performance can be closely scrutinised without introducing errors due to handling of the amplicon [49]. In addition, real-time PCR has proven to be cost-effective on a per-run basis, when implemented in a high-throughput laboratory [50], particularly when replacing conventional, culture-based approaches to microbial detection.

In the remainder of this review, the theory behind real-time PCR will be discussed. Additionally, its rapidly expanding use in the study of human infectious disease will provide an example of its acceptance and effectiveness in the diagnostic microbiology laboratory.

AMPLICON DETECTION

It is the detection process that discriminates real-time PCR from conventional PCR assays. There is a range of chemistries currently in use which can be broadly categorised as specific or non-specific for the amplicon's sequence [51]. These have recently been reviewed in detail [52]. Several additional reporter systems have since been described, and these will be discussed below; however, few applications have been described for the specific detection and genotyping of microbes.

While the most common oligoprobes are based on traditional nucleic acid chemistry, the peptide nucleic acid (PNA) is becoming a more popular choice for oligonucleotide backbones. The PNA is a DNA analogue that is formed of neutral repeated *N*-(2-aminoethyl) glycine units instead of negatively charged sugar phosphates [53]. However, the PNA retains the same sequence recognition properties as DNA.

In general, however, the specific and non-specific fluorogenic chemistries detect amplicon with the same sensitivity [39].

LINEAR OLIGOPROBES

The use of a pair of adjacent, fluorogenic hybridisation oligoprobes was first described in the late 1980s [45,54], and, now known as 'HybProbes', they have become the manufacturer's chemistry of choice for the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany), a capillary-based, microvolume fluorimeter and thermocycler with rapid temperature control [39,55]. The upstream oligoprobe is labeled with a 3′ donor fluorophore (fluorescein isothiocyanate, FITC), and the downstream probe is commonly labelled with either a LightCycler Red 640 or Red 705 acceptor fluorophore at the 5′-terminus, so that when both oligoprobes are hybridised, the two fluorophores are located within 10 nucleotides of each other.

Most recently described fluorogenic oligoprobes fall into the linear class of oligoprobe. The recently described double-stranded oligoprobes function by displacement hybridisation (Fig. 1a) [56]. In this process, a 5′ fluorophore-labelled oligonucleotide is, in its resting state, hybridised with a complementary, but shorter, quenching DNA strand that is 3′ end-labeled with an NFQ. When the full-length complementary

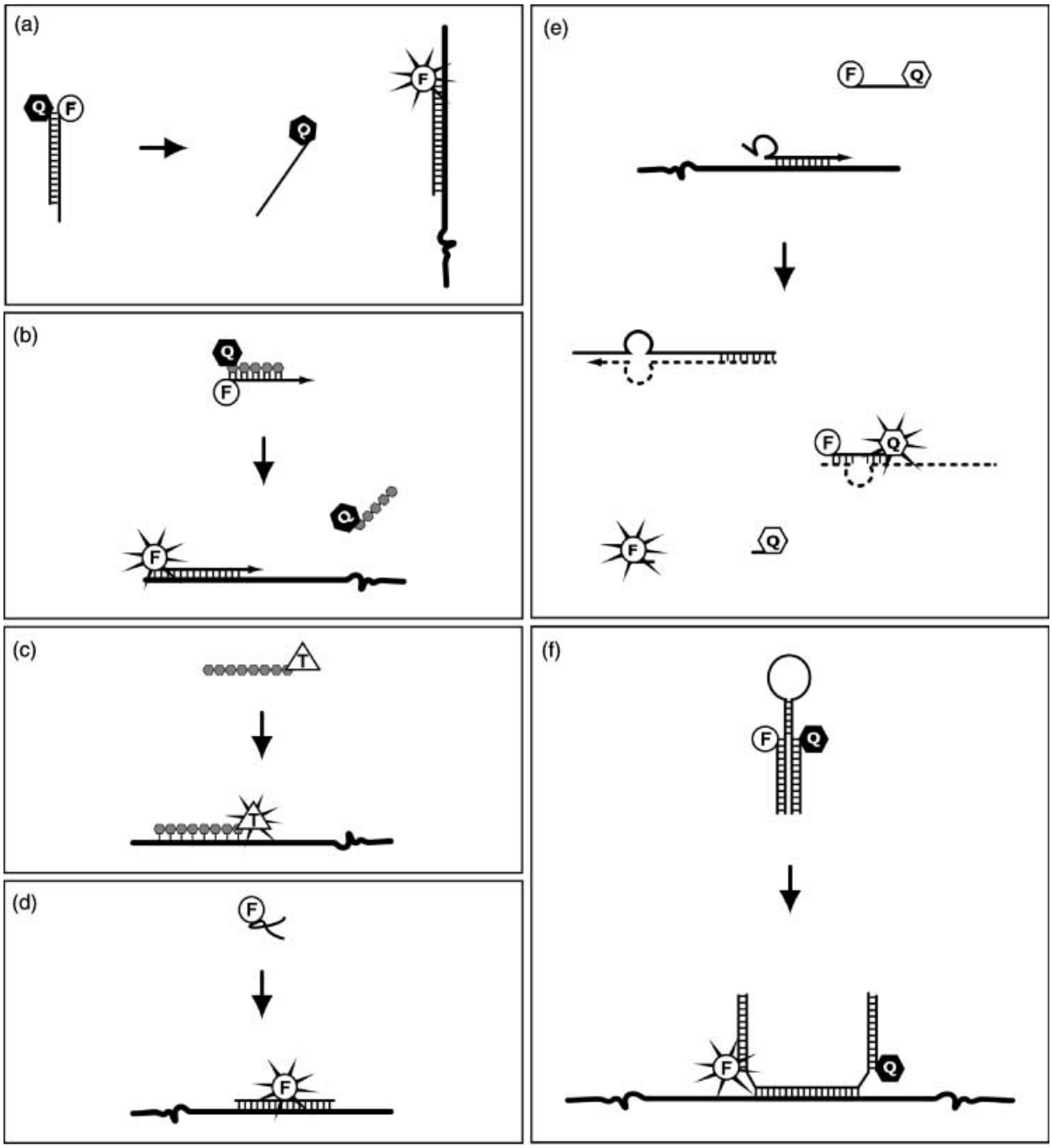

Fig. 1. Oligoprobe chemistries. (a) Displacement probes. The shorter NFQ-labeled strand (Q; filled pentagon) is displaced when the fluorophore-labelled (F; open circle) strand hybridises to the specific and longer amplicon. (b) Q-PNA primers. Quenching is achieved in the absence of specific template by a short NFQ-labelled PNA molecule designed to hybridise with the fluorophore-labelled primer. (c) Light-up probes. These PNA probes fluoresce in the presence of a hybridised DNA strand due to their asymmetric thiazole orange fluorophore (T; open triangle). (d) HyBeacons. In close proximity to DNA, as occurs upon hybridisation with the specific amplicon, the fluorophore emits fluorescence. (e) DzyNA primers. When the primer is duplicated by the complementary strand (dashed line), a DNAzyme is created. In the presence of a complementary, dual-labelled oligonucleotide substrate, the continuously amplified DNAzyme will specifically cleave the template between the fluorophore and quencher (Q; open pentagon), releasing the labels and allowing fluorescence to occur. (f) Tripartite molecular beacons. The fluorophore is removed from the NFQ's influence upon opening of the hairpin because of hybridisation to specific amplicon, permitting fluorescence.

sequence in the form of an amplicon is present, the reporter strand will preferentially hybridise to the longer amplicon, disrupting the quenched oligoprobe duplex and permitting the fluorophore to emit its excitation energy directly.

This technique can also be used with a fluorophore-labelled primer, and, due to the added stringency of the complementary strand, the system acts as its own 'hot-start', as was shown using an NFQ-labeled PNA [57] strand (Q-PNA) (Fig. 1b) [58]. In this system, the quenching probe is bound to unincorporated fluorogenic primer such that the NFQ and fluorophore are adjacent, resulting in a quenched system. Once the dsDNA amplicon is created by primer extension, however, the Q-PNA is displaced, and the fluorophore can fluoresce. The PNA backbones cannot be extended or hydrolysed by a DNA polymerase.

The light-up probe is also a PNA to which the asymmetric cyanine fluorophore thiazole orange is attached (Fig. 1c) [59]. When hybridised with a nucleic acid target, as either a duplex or triplex, the fluorophore becomes strongly fluorescent. These oligoprobes do not interfere with the PCR or require conformational change, they are sensitive to single nucleotide mismatches and, because a single reporter is used, they allow the direct measurement of fluorescence instead of the measurement of a change in fluorescence between two fluorophores [59,60]. However, non-specific fluorescence has been reported during extended cycling [61].

The HyBeacon is a single linear oligonucleotide internally labelled with a fluorophore that emits an increased signal upon formation of a duplex with the target DNA strand (Fig. 1d) [62,63]. The HyBeacon is labelled at the 3′-terminus with a phosphate or octanediol molecule to prevent *Taq*-mediated extension. This technique is used with all the non-incorporating nucleotide-based oligoprobe chemistries used in real-time PCR to ensure that they do not function as a primer. This chemistry does not require destruction, interaction with a second oligoprobe or secondary structure changes to produce a signal, and it is relatively cheap and simple to design.

DUAL-LABELLED OLIGOPROBES

In the early 1990s, an innovative approach involved nick-translation PCR in combination with dual-fluorophore-labelled oligoprobes was introduced [26]. In the first truly homogeneous assay of its kind, a fluorophore was added to the 5′-terminus and another to the middle of a sequence-specific oligoprobe. When in such close proximity, the 5′ reporter fluorophore (6-carboxy-fluoroscein; FAM) transferred laser-induced excitation energy by FRET to the 3′ quencher fluorophore (6-carboxy-tetramethyl-rhodamine; TAMRA). The oligoprobe hybridised to its template prior to the extension step, and the fluorophores were subsequently released during the primer extension step as a result of the 5′ to 3′ endonuclease activity of a suitable DNA polymerase. Once the labels were separated, the reporter's emissions were no longer quenched, and the instrument monitored the resulting fluorescence. Today, these oligoprobes are labelled at each terminus and are called 5′ nuclease, hydrolysis or TaqMan oligoprobes. The nuclease oligoprobe is the manufacturer's chemistry of choice for the ABI Prism sequence detection systems.

A modification of the 5′ nuclease chemistry has resulted in the minor groove binding (MGB) oligoprobes [64]. This chemistry, commercially called the Eclipse oligoprobes, replaces the TaqMan oligoprobe's standard TAMRA quencher with a proprietary NFQ and incorporates a molecule that hyperstabilises the oligoprobe–target duplex by folding into the minor groove of the dsDNA [65,66]. A fluorophore is attached to the 3′ end, and in the unbound state the oligoprobe assumes a random coil configuration that is efficiently quenched. This chemistry allows the use of very short (12–17-nucleotide) oligoprobes because of a 15–30 °C rise in their T_M resulting from the interaction of the MGB with the DNA helix. These short oligoprobes are ideal for detecting single-nucleotide polymorphisms (SNPs), because they are more significantly destabilised by nucleotide changes within the hybridisation site than are larger oligoprobes.

Another dual-labelled oligonucleotide sequence has been used as the signal-generating portion of the DzyNA-PCR system (Fig. 1e) [67]. Here, the reporter and quencher molecules are separated following specific cleavage of the oligonucleotides holding them in close proximity. This cleavage is performed by a DNAzyme, which is created during the PCR as the complement of an antisense DNAzyme sequence included in the 5′ tail of one of the primers. Upon cleavage, the fluorophores are released, allowing

the production of fluorescence in an identical manner to a hydrolysed TaqMan oligoprobe.

HAIRPIN OLIGONUCLEOTIDES

Molecular beacons were the first hairpin oligoprobes to be used in real-time PCR. The molecular beacon's fluorogenic labels are positioned at the termini of the oligoprobe. The labels are held in close proximity by distal stem regions of homologous base pairing deliberately designed to create a hairpin structure. The closed hairpin is quenched due either to FRET or direct collision transfer of energy occurring at the molecular level as a consequence of the intimate proximity of the labels [68]. In the presence of a complementary sequence, designed to occur within the bounds of the primer binding sites, the oligoprobe will hybridise, shifting into an open configuration. The fluorophore is now spatially removed from the quencher's influence, allowing fluorescent emissions to be monitored [69]. This structural change occurs in each cycle, increasing in cumulative intensity as the amount of specific amplicon increases. The quencher, DABCYL (4-(4'-dimethylamino-phenylazo)-benzene), differs from that described for the nuclease oligoprobes because it is an NFQ.

Recently, tripartite molecular beacons have been added to this class of fluorogenic chemistry (Fig. 1f) [70]. These oligoprobes have been designed to fulfill a need for suitably high-throughput chemistries and they combine a molecular beacon's hairpin with long or unlabelled single-stranded arms, each designed to hybridise to an oligonucleotide labelled with either a fluorophore or an NFQ. The system is quenched in the hairpin state due to the close proximity of the labels, but fluorescent when hybridised to the specific amplicon strand. Because the function of these oligoprobes depends upon correct hybridisation of the stem and two oligoprobes, their accurate design is crucial [8].

Finally, a self-quenching hairpin primer has recently been described which is commercially entitled the light upon extension (LUX) fluorogenic primer [71]. This chemistry is dark in the absence of specific amplicon, through the natural quenching ability of a carefully placed guanosine nucleotide. The natural quencher is brought into close proximity with the FAM or JOE 5' 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluoroscein fluorophore via a stretch of 5' and 3' complementary sequences. In the presence of specific target, the primer hybridises, opening the hairpin and permitting fluorescence from the fluorophore.

MICROBIAL QUANTITATION

Although the terminology is often confused, real-time PCR does not inherently imply quantitative PCR. To quantify the amount of template present in a sample, thought must be given to the type and number of controls required. Standards are used to allow calculation of the amount of template present in a patient sample, while internal controls (ICs) are mostly used to determine the occurrence of false-negative reactions, examine the ability to amplify from a preparation of nucleic acids, and, more rarely in real-time PCR, as a standard for quantitation. Certainly, the reliability of quantitative PCR methods is intimately associated with the choice and quality of the assay controls [72,73].

No matter what control is chosen, it is imperative to accurately determine its concentration [74] and to ensure that ICs are added at suitable levels in order to prevent extreme competition with the wild-type template for reagents [75]. The use of a spectrometer is inadequate for quantitating a control molecule [76]; however, in combination with an experimental and statistical analysis, the reliability of the values is greatly enhanced [77–81]. Finally, one must remember that the results of quantitation using a molecular control need to be expressed relative to a suitable biological marker, e.g., in terms of the volume of plasma, the number of cells or the mass of tissue or genomic nucleic acid, thus allowing comparability between assay results and testing sites [82].

Standards for quantitation

Most commonly, an exogenous control is created using a cloned amplicon, a portion of the target organism's genome, or simply the purified amplicon itself [83]. This control forms the basis of an external standard curve created from the data produced by the individual amplification of a dilution series of exogenous control. The concentration of an unknown, which is amplified in the same reaction, but in a separate vessel, can then be found from the standard curve. While the

external standard curve is the more commonly described quantitative approach, it frequently suffers from uncontrolled and unmonitored inter-vessel variations. Some platforms have overcome this issue by including a capacity to detect and correct for variation in the emissions of a non-participating, or 'passive', internal reference fluorophore (6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine; ROX). The corrected values, obtained from a ratio of the emission intensity of the fluorophore and ROX, are called RQ^+. To further control amplification fluctuations, the fluorescence from a 'no-template' control reaction (RQ^-) is subtracted from RQ^+, resulting in the ΔRQ value that indicates the magnitude of the signal generated for the given PCR [84]. Assays that lack this capacity are more appropriately described as semiquantitative.

Internal controls (ICs)

The use of an IC was described in the earliest of PCR experiments as an important quality control [85,86], particularly when performing competitive quantitation. When such a control is added before template purification (extraction control) or amplification (amplification control), it is called an exogenous IC, since it does not occur naturally within the nucleic acid preparation, but is co-amplified within the same reaction. Ideally, the IC should hybridise to the same primers, have an identical amplification efficiency [74,87], and contain a discriminating feature such as a change in its length [72,72,75,88,89] or, more commonly in today's oligoprobe-based methods, a change in the sequence [73,90] of the wild-type target [91,92]. However, IC templates that bind different primers or have different amplification efficiencies can still prove useful as standards for semi-quantitative PCR or relative quantitation.

An endogenous control is a template that occurs naturally within the specimen being examined. Housekeeping genes often fulfill this role, and they have been successfully used to quantitate gene expression by RT-PCR and monitor the integrity of a template after its purification [85]. When endogenous controls are used for the quantitation of RNA, it is essential that the housekeeping gene is minimally regulated and exhibits a constant and cell cycle-independent basal level of transcription [93]. This is not the case for some commonly used genes such as β-actin, whereas studies have shown that an 18S rRNA target meets the desired criteria [93,94].

Relative vs. absolute quantitation

The amount of template in a sample can be described either relatively or absolutely. Relative quantitation is the simpler approach, and describes changes in the amount of a target sequence compared to its level in a related matrix or within the same matrix by comparison to the signal from an endogenous or other reference control. Absolute quantitation is more demanding but states the exact number of nucleic acid targets present in the sample in relation to a specific unit, making it easier to compare data from different assays and laboratories [7,95]. Absolute quantitation may be necessary when there is a lack of sequential specimens to demonstrate a relative change in microbial load, or when no suitably standardised reference reagent is available.

A highly accurate approach used for absolute quantitation by conventional PCR utilises competitive coamplification of one or a series of ICs of known concentration with a wild-type target nucleic acid of unknown concentration [96–99]. However, conventional competitive quantitation is technically demanding, requiring significant development and optimisation compared to quantitation by real-time PCR, which is better suited to the quick decision-making required in a clinical environment [100–102]. Software with the ability to calculate the concentration of an unknown by comparing real-time PCR signals generated by a coamplified target and IC is rare but emerging [7]. In addition, new or improved formulae are appearing which aim to make quantitation more reliable and simpler [103].

Acquisition of fluorescence data

Fluorescence data generated by real-time PCR assays are generally collected from PCR cycles that occur within the linear amplification portion of the reaction, where conditions are optimal and the fluorescence accumulates in proportion to the amplicon [52] (Fig. 2). This is in contrast to signal detection from the endpoint of the reaction, where the final amount of amplicon may have been affected by inhibitors, poorly optimised reaction conditions or saturation effects due to the

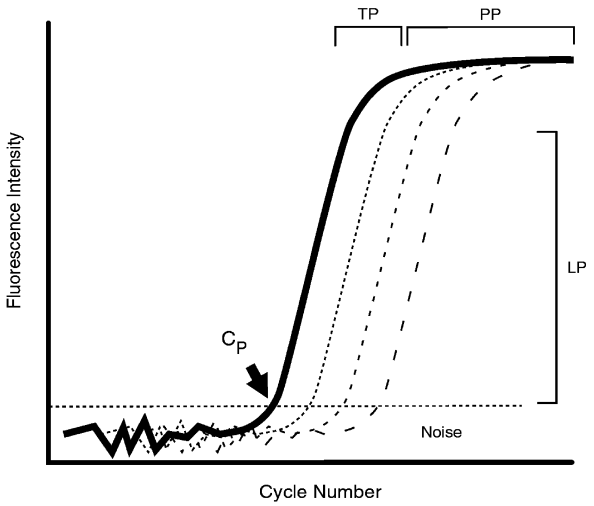

Fig. 2. Kinetic analysis. The ideal amplification curve of a real-time PCR (solid), when plotted as fluorescence intensity against the cycle number, is a sigmoidal curve. Early amplification cannot be viewed because the emissions are masked by the background noise. However, when enough amplicon is present, the assay's exponential progress can be monitored as the rate of amplification enters a linear phase (LP). Under ideal conditions, the amount of amplicon increases at a rate of one $\log_{10}$ every 3.32 cycles. As primers and enzyme become limiting, and products inhibitory to the PCR and overly competitive to oligoprobe hybridisation accumulate, the reaction slows, entering a transition phase (TP) and eventually reaching a plateau phase (PP) where there is little or no increase in fluorescence. The point at which the fluorescence surpasses the noise threshold (dashed horizontal line) is called the threshold cycle or crossing point (C_T or C_P; indicated by an arrow), and this value is used in the calculation of template quantity during quantitative real-time PCR. Also shown are curves representing a titration of template (dashed curves), consisting of decreasing starting template concentrations, which produce higher C_T or C_P values, respectively. Data for the construction of a standard curve are taken from the LP.

presence of excess double-stranded amplicon. In fact, at the endpoint there may be no relationship between the initial template and final amplicon concentrations. Because the emissions from fluorescent chemistries are temperature-dependent, data are generally acquired only once/cycle, at the same temperature [55].

The fractional cycle number at which the real-time fluorescence signal mirrors progression of the reaction above the background noise is used as an indicator of successful target amplification [104]. Most commonly, this is called the threshold cycle (C_T), but a similar value is described for the LightCycler, and the fractional cycle is called the crossing point (C_P). The C_T is defined as the PCR cycle in which the gain in fluorescence generated by the accumulating amplicon exceeds ten standard deviations of the mean baseline fluorescence, using data taken from cycles 3–15 [105]. The C_T and C_P are proportional to the number of target copies present in the sample [29] and are assumed to represent equal amounts of amplicon present in each tube or capillary, since the C_T and C_P values represent the fractional cycle number for each sample at a single fluorescence intensity value. In practice, the C_T and C_P are calculated after the definition of a noise band that excludes data from early PCR cycles that cannot be distinguished from background noise. The final C_T and C_P values are the fractional cycles at which a single fluorescence value (usually at or close to the noise band) intersects each sample's plotted PCR curve [104] (Fig. 2). The accuracy of the C_T or C_P depends upon the concentration and nature of the fluorescence-generating component, the amount of template initially present, the sensitivity of the platform, and the platform's ability to discriminate specific fluorescence from background noise.

Improved quantitation using real-time PCR

Significant improvements in the quantitation of microbial load by real-time PCR result from the detection system's enormous dynamic range, which can accommodate at least eight $\log_{10}$ copies of nucleic acid template [92,100,106–114]. The broad dynamic range avoids the need for predilution of an amplicon before detection, or the need to repeat an assay using a diluted sample because a preliminary result falls outside the limits of the assay. Both of these problems occur commonly when using conventional endpoint PCR assays for quantitation, as their detection systems are unable to encompass the products of high template loads while maintaining adequate sensitivity [113,115–117]. The flexibility of real-time PCR is further demonstrated by its ability to detect one target in the presence of a vast excess of another target during duplexed assays [109].

Real-time PCR is also a particularly attractive alternative to conventional PCR for the study of microbial load because of its low inter-assay and intra-assay variability [100,112,118] and its equivalent or improved sensitivity compared to microbial culture, or conventional single-round and nested PCR [17,100,110,119–126]. Real-time PCR has been reported to be at least as sensitive as

Southern blot, still considered by some as the gold standard for probe-based hybridisation assays [122].

MICROBIAL GENOTYPING

Although nucleotide sequencing is still the gold standard for characterising unknown nucleic acids, it is a relatively lengthy process. The development of real-time PCR has partially addressed this failing by providing a tool capable of routine detection of characterised mutations, insertions or deletions.

Most fluorescent chemistries used for real-time PCR do not rely upon a destructive process to generate a signal. Therefore, they may be able to perform a genotyping role at the completion of the PCR. The SYBR green and HybProbe chemistries are most commonly used to perform these analyses; however, the double-stranded and light-up oligoprobes and HyBeacons should also function in this role. Other chemistries, such as the TaqMan and Eclipse oligoprobes and hairpin oligonucleotides, discriminate these nucleotide changes using two sets of oligoprobes to differentiate the wild-type from the altered sequences. While this is a perfectly legitimate and functional approach to genotyping by real-time PCR, the extra fluorogenic oligonucleotides increase the overall cost of the assay. Additionally, the number of different microbes that can be discriminated during multiplex real-time PCR is reduced, since two fluorophores must be assigned to analyse each microbe. The occurrence of a mismatch between a hairpin oligonucleotide and its target has a greater destabilising effect on the duplex than the introduction of an equivalent mismatch between the target and a linear oligoprobe. This is because the hairpin structure provides a highly stable alternative configuration. Therefore, hairpin oligonucleotides are more specific than the more common linear oligoprobes, making them ideal candidates for detecting SNPs [68].

Genotyping data are obtained after the completion of the PCR, and therefore represent an endpoint analysis. The amplicon is denatured and rapidly cooled to encourage the formation of fluorophore and target strand complexes. The temperature is then gradually raised, and the fluorescence from each vessel is continuously recorded. The detection of sequence variation using fluorescent chemistries relies upon the destabilisation incurred as a result of the change(s). The non-specific chemistries reflect these changes in the context of the entire dsDNA amplicon, requiring the dissociation of fluorogenic molecules from the dsDNA, which only occurs upon melting of the duplex. The sequence changes have a different impact upon the specific fluorogenic chemistries, altering the expected T_M in a manner that reflects the particular nucleotide change. The resulting rapid decrease in fluorescence using either approach can be presented as a 'melt peak' using software capable of calculating the negative derivative of the fluorescence change with temperature (Fig. 3).

Importantly, different nucleotide changes destabilise hybridisation to different degrees, and this can be incorporated into the design of genotyping assays to ensure maximum discrimination between melt peaks. The least destabilising mismatches include G (G:T, G:A and G:G), whereas the most destabilising include C (C:C, C:A and C:T) [127].

MULTIPLEX REAL-TIME PCR

Multiplex PCR uses one or more primer sets to potentially amplify multiple templates within a single reaction [128,129]. However, its use in real-time PCR has led to confusion in the traditional terminology. Multiplex real-time PCR more commonly refers to the use of multiple fluorogenic oligoprobes for the discrimination of amplicons that may have been produced by one or several primer pairs. The development of multiplex real-time PCR has proven problematic because of the limited number of fluorophores available [26] and the frequent use of monochromatic energising light sources. Although excitation by a single wavelength produces bright emissions from a suitably receptive fluorophore, the number of fluorophores that can be excited by that wavelength is limited [130].

The discovery and application of the non-fluorescent quenchers has made available some wavelengths that were previously occupied by the emissions from the early quenchers themselves. This should permit the future inclusion of a greater number of spectrally discernable oligoprobes/reaction, and highlights the need for a single non-fluorescent quencher that can quench a broad range of emission wavelengths (e.g.,

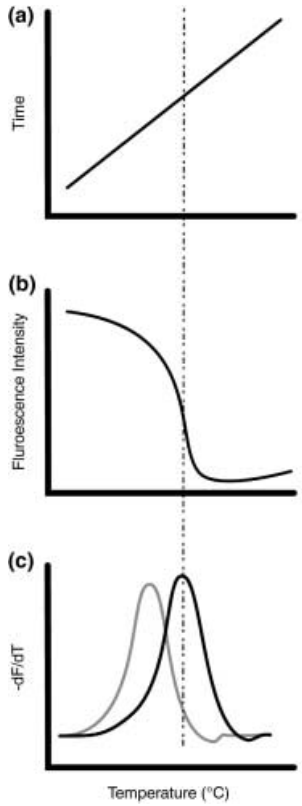

Fig. 3. Fluorescence melting curve analysis. At the completion of a real-time PCR using a fluorogenic chemistry, the reaction can be cooled to a temperature below the expected T_M of the oligoprobes and then heated to above 90°C at a fraction of a degree/second (a). During heating, the emissions of the reporter or acceptor fluorophore can be constantly acquired (b). Software calculates the negative derivative of the fluorescence with temperature, producing a clear melt peak that indicates the T_M of the oligoprobe–target melting transition (black peak; c) or the T_D of melting dsDNA. When one or more nucleotide changes are present, the T_M or T_D is shifted (grey peak). This shift is reproducible and can be used diagnostically to genotype microbial templates.

400–600 nm). The impressive electron-donating properties of guanosine make it an ideal natural quencher, and its use has contributed to the growing number of assays that only require a single fluorophore/target [131].

Early real-time PCR systems contained optimised filter sets to minimise overlap of the emission spectra from the fluorophores. Despite this, the number of fluorophores that could be combined and clearly distinguished was limited. More recent real-time PCR platforms have incorporated either multiple light-emitting diodes, or a tungsten light source that emits over a wide range of wavelengths. When these platforms also incorporate high-quality optical filters, it is possible to use many of the current real-time PCR detection chemistries on the one machine. Unfortunately some platforms are not suitably constructed. Even if these improvements are included, the platform can still only perform four-colour oligoprobe multiplexing, and one colour is ideally set aside for use as an IC. Some real-time PCR designs have made use of conserved single or multiple nucleotide changes among similar templates to allow their differentiation by concurrent changes to the oligoprobe's T_M or the amplicon's T_D [132,133]. Combining the use of multiple fluorophores with the discrimination of additional targets by temperature allows the identification of a significantly larger number of amplicon targets [134]; however, this combined approach has not been applied to the diagnosis of infectious disease on a significant scale [135], possibly because of the sequence variation among many microbial genes [119,136–139]. Far more commonly, this approach has been used for the detection of human genetic diseases, where as many as 27 possible nucleotide substitutions have been detected using only one or two fluorophores [140–147].

To date, there have been only a handful of diagnostic microbial assays that can truly co-amplify and discriminate more than two fluorophores. An impressive multiplex, real-time PCR protocol discriminated between four retroviral target sequences [148]; however, conventional multiplex PCR using endpoint detection has easily discriminated between more than five different amplified sequences, indicating a greater degree of flexibility [149–154].

Future development of novel chemistries and improved real-time instrumentation and software should significantly improve the ability to multiplex fluorophores for enhanced real-time PCR assays. Perhaps a chimera of real-time PCR and microarray technology, in combination with microfluidic devices, may advance all three technologies to a point where the desired number of templates could be easily amplified and discriminated.

SPECIFIC APPLICATIONS FOR MICROBIOLOGY

Real-time PCR assays have been extremely useful for studying microbial agents of infectious disease, where they have helped to clarify many

disease processes. Most of the assays presented in the literature have increased the frequency of microbial detection compared to non-PCR techniques, making the implementation of real-time PCR attractive to many.

Of course, real-time PCR has also proven valuable for basic microbiological research, where its ability to amplify template from a wide array of sample types (Table 1) has made it an ideal system for application across the various microbiological disciplines [155]. Increasingly, these applications are difficult to review, due to their use as a tool within, rather than the focus of, a published study.

Viruses

Within microbiology, the application of real-time PCR has had the biggest impact upon the field of virology, where studies have qualitatively investigated the role of viruses in a range of human diseases [156]. Also, epidemiological studies of co-infections have been improved by these molecular techniques, which can reliably measure the amount of two nucleic acid targets present within a single sample [119,157,158]. Real-time PCR has also improved the discrimination of multiple viral genotypes within a single reaction vessel [159] and provided an alternative to morbidity and mortality assays for virus detection. An example is Newcastle disease virus, which exists as two radically different pathogenic phenotypes caused by small nucleotide changes that can be easily detected using fluorescence melting curve analysis to reveal the genetic pathotype of the strain [160].

Table 1. An incomplete list indicating the extraordinary variety of sample types from which nucleic acids can be successfully prepared, amplified and detected using real-time PCR assays

Nucleic acid origins	References
Plants	[293]
Animals	[111,260]
Urban sludge	[110]
Microbial culture	[177,234,240,253,254,266,267, 280,284,292,294]
Solid tissues	[172,198,238,256–258,271,288, 295]
Cerebrospinal fluid	[136,190,192,194,248]
Peripheral blood mononuclear cells	[183]
Bone marrow	[120]
Whole blood	[179,291]
Plasma	[118]
Serum	[171,172,180,279,287]
Swabs	[192,259,296]
Bronchoalveolar lavage	[243,246]
Amniotic fluid	[286]
Saliva and sputum	[158,233]
Faeces	[264,285]
Urine	[12,155,178]

Direct and indirect links between viral infection and chronic conditions such as sarcoma [121,161–164], carcinoma [122,165], cervical intra-epithelial neoplasia [166–168] and lymphoproliferative disorders [169,170] can be relatively easily studied using real-time PCR. Other studies have described the presence of flaviviruses [106,126, 171–176], hepadnaviruses [113,115,177], herpesviruses [30,40,100,102,107–109,116,119,121,122,137, 155,158,165,178–187], orthomyxoviruses [125], parvoviruses [92], papovaviruses [32,139,159], paramyxoviruses [124,160,188], pestiviruses [189], picornaviruses [110,111,190–195], poxviruses [196], retroviruses [118,123,197–200], rhabdoviruses [201] and TT virus [202].

A significant number of studies have used PCR to detect viral load, and have proved its usefulness as an indicator of the extent of active infection, interactions between virus and host, and the changes in viral load as a result of antiviral therapies, all of which can play a role in the treatment regimen selected [203–205]. Conventional quantitative PCR has already proven that the application of nucleic acid amplification to the monitoring of viral load provides a useful marker of disease progression and the efficacy of antiviral compounds [97,204,206–210]. Because disease severity and viral load are linked, the use of real-time PCR quantitation has proven beneficial when studying the role of viral reactivation or persistence in the progression of disease [40,102,107,108,119,158,165,171,183,184,187,211, 211–216]. Alterations to a microbe's tropism or its replication, and the effects that these changes have on a host cell, can also be followed using real-time PCR [217–219].

The role of highly sensitive and rapid real-time PCR assays in the thorough assessment of viral gene therapy vectors before their use in clinical trials has become an important one. Nuclease oligoprobes have been most commonly used for these studies, which assess the biodistribution, function and purity of the novel 'drug' preparations [199,220–225].

Likewise, the study of new and emerging viruses has been ideally complemented by the use of homogeneous real-time PCR assays as tools to demonstrate and strengthen epidemiological links between unique viral sequences and the

clinical signs and symptoms experienced by patients [124,126,201,226–230].

The speed and flexibility of real-time PCR has also proven useful for commercial interests who require exquisite sensitivity to screen for microbial contamination within large-scale reagent preparations produced from eukaryotic expression systems [231,232].

Bacteria

The benefits to the patient from rapid real-time PCR assays are most notable when applied to the detection of bacteria. The results can quickly inform the clinician as to the infection status of the patient, allowing a more specific and timely application of antibiotics. This can limit the potential for toxicity due to shotgun treatment regimens, reduce the duration of a hospital stay and prevent the improper use of antibiotics, thus minimising the potential for resistant strains to emerge.

Broad applications of real-time PCR can augment or replace traditional culture or histochemical assays, as was seen with the creation of a molecular assay capable of classifying bacteria in the same way as a Gram stain [233]. However, specific bacterial species are more frequently the focus for real-time PCR assays, especially when long culture times can be replaced by rapid and specific gene detection. *Leptospira* genospecies, *Mycobacterium* and *Propionibacterium* spp., *Chlamydia* spp., *Legionella pneumophila* and *Listeria monocytogenes* have all been detected and in some cases quantitated with the use of real-time PCR assays [41,234–246].

The detection of *Neisseria gonorrhoeae* has benefited from real-time PCR, particularly in the role of a confirmatory test when the specificity of commercial assays fails [247]. This example highlights the need for care when choosing a bacterial PCR target, especially when that target exists on a plasmid that is exchanged among other bacteria, providing potentially confusing diagnostic results. *Neisseria meningitidis* causes meningococcal disease, and real-time PCR has proven to be a powerful tool that can be quickly developed for the rapid discrimination of currently circulating pathogens [248].

The detection and monitoring of antibiotic resistance among clinical isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Helicobacter pylori*, *Enterococcus faecalis* and *Enterococcus faecium* has also benefited from real-time applications [249–258]. Additionally, the understanding and treatment of fulminant diseases such as meningitis, sepsis, inflammatory bowel disease and food-poisoning caused by characterised bacteria such as the group B streptococci and *Mycobacterium* spp., *Escherichia coli* and *Bacteroides vulgatus* [259–265] have been enhanced by the speedy return of results, which also aids tracking of microbial outbreaks to their source.

Real-time PCR has made possible the rapid quantitation and differentiation of some of the more exotic pathogenic bacteria, such as the tick-borne spirochete *Borrelia burgdorferi* [266–268] and the methanotropic bioremediating *Methylocystis* spp. [269].

The involvement of treponemes in the development of periodontal disease has been studied using 5′ nuclease chemistry, revealing a microbial role in every stage [270]. In addition, measurement of the bacterial load of *Tropheryma whipplei* has allowed the discrimination of environmental contamination and low-level colonisation from active infection [271].

More recently, there has been an explosion of literature indicating that real-time PCR is the tool of choice for the rapid detection of microbes used as agents of biological warfare. In some cases, the assays have allowed rapid discrimination of weaponised pathogens from the harmless laboratory-adapted or vaccine-related strains. At the forefront of the available literature are assays to detect *Bacillus anthracis* spores and the bacterium's virulence-encoding plasmids or chromosomal markers [272,273,273–277]. Conventional assays may take 48 h to complete, and, for obvious reasons, this is an unacceptable lag period.

Fungi, parasites and protozoans

The smallest number of applications have been related to the study of fungal, parasitic and protozoan pathogens of humans. Nonetheless, real-time PCR assays have significantly contributed to the general diagnosis of invasive disease caused by *Aspergillus fumigatus* and *Aspergillus flavus* [278,279]. Also, monitoring the transcription levels of certain *Aspergillus nidulans* transporter genes has provided important information about their role in multiresistance [280]. In addition, real-time assays have been used when investi-

gating buildings for the presence of potentially harmful levels of toxigenic fungal spores, or conidia, such as those produced by *Stachybotrys chartarum* [281–283].

Cryptosporidium parvum oocysts and the spores from *Encephalitozoon* spp. have been successfully genotyped or speciated using real-time PCR, which has significantly improved on laboratory diagnosis using microscopy and histochemical staining, especially for low concentrations of excreted material [284,285].

Rapid serological detection of *Toxoplasma gondii* is often hampered by the presence of the parasite in patients who are immunocompromised. Additionally, the length of time required for traditional culture or mouse inoculation is excessive. Therefore, rapid molecular methods have vastly improved the detection of this microbe [120,286]. Additionally, this technology is useful for the study of *T. gondii* responses to antimicrobial therapies [287].

Detection of malarial parasites using a mouse model in combination with real-time PCR has improved result turnaround time and meant that parasite load data can be generated [288,289]. Real-time PCR has also proven useful for direct in-vivo detection and quantitation of malarial parasites with a high level of sensitivity [290,291], in addition to monitoring the stage-specific maturation of *Plasmodium falciparum* via the transcription of specific genes [292].

CONCLUSIONS AND SUMMARY

Microbiology is ideally suited for the benefits of sensitivity and rapidity that PCR has brought to the research laboratory. However, the advent of real-time PCR has further improved the role of PCR in the high-throughput environment by adding a detection system capable of enormous dynamic range, homogeneity of amplification and detection, and the ability to genotype an amplified nucleic acid without the need for additional steps. Unfortunately, many of the genotyping applications have been trialled first in the field of human genetics, where there is frequently a more abundant source of template, and the genetic changes, once characterised, remain constant. Nonetheless, this review has highlighted the general acceptance of real-time PCR in the research and diagnostic microbiology laboratory, and its popularity is continuing to expand.

Advances in the development of fluorophores, nucleotide labelling and the novel application of oligoprobe hybridisation have provided real-time PCR technology with a broad enough commercial base to promote its usefulness to the wider non-research scientific community. Robotic nucleic acid extraction and liquid-handling systems, combined with rapid thermal cyclers and instrumentation capable of detecting and differentiating multiple amplicons using many of the chemistries described in this and other reviews, make real-time PCR an attractive and viable proposition for the routine diagnostic laboratory. Many laboratories rely upon tissue culture to isolate microbial agents of infectious disease, in combination with serological methods to further confirm the identity of the isolates or to monitor a patient's immune response to an infectious agent. Such methods, while providing an important source of information about unknown and emerging pathogens, may take a prolonged and clinically significant amount of time to complete.

According to the literature, the most widely used fluorogenic probe format is the 5′ nuclease oligoprobe, although that is most likely due to its commercial maturity. The rate of publications describing other methods, especially those utilising the LightCycler in combination with a pair of HybProbes, is significant and changing the balance rapidly, especially in the area of microbial detection and genotyping. There are also more virus-detecting real-time PCR applications described in the literature than for any other microbe. The more recently developed oligoprobe chemistries have been used in only a few innovative applications, but they will be better understood as their benefits and limitations are more widely described, and hopefully they will allow a greater variety of options for microbial genotyping.

Recent developments in multiplex real-time PCR have suggested a future in which easy identification, genotyping and quantitation of microbial targets in single, rapid reactions will be commonplace. Of course, real-time PCR is by no means restricted to microbiology, as significant achievements have already been made in the area of human genetic diagnostics, applying all the benefits of real-time PCR to enhance the detection of genetic disease. However, the technology is only as reliable as the accompanying controls and associated quality assurance programmes. This includes the quality of standards, the use of

suitably controlled standard curves, and the need to fully optimise, validate and evaluate each and every new assay against previously standardised assays. Without such care, real-time PCR will provide fast but inaccurate data to the clinician, who will surely come to rely upon such assays, as they represent a growing proportion of the result-generating tests within the diagnostic microbiology environment. In addition, commercial interests will undoubtedly play an expanding role in determining which technologies enter into the mainstream.

Perhaps in combination with micro- and macroarray technology and emerging microfluidic devices, real-time PCR assays that can discriminate as many targets as desired, while producing quantitative data at a greatly increased speed, will consolidate fluorogenic nucleic acid amplification as a routine and incredibly powerful tool for the laboratory of tomorrow.

ACKNOWLEDGMENTS

This work was supported by a Royal Children's Hospital Foundation Grant (922-034), which was sponsored by the Woolworth's 'Care for Kids' campaign. The author wishes to thank Dr Katherine Arden for critical review of this manuscript.

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