

SURVEY AND SUMMARY

Real-time PCR in virology

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ABSTRACT

The use of the polymerase chain reaction (PCR) in molecular diagnostics has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory. Real-time PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. There are currently five main chemistries used for the detection of PCR product during real-time PCR. These are the DNA binding fluorophores, the 5' endonuclease, adjacent linear and hairpin oligoprobes and the self-fluorescing amplicons, which are described in detail. We also discuss factors that have restricted the development of multiplex real-time PCR as well as the role of real-time PCR in quantitating nucleic acids. Both amplification hardware and the fluorogenic detection chemistries have evolved rapidly as the understanding of real-time PCR has developed and this review aims to update the scientist on the current state of the art. We describe the background, advantages and limitations of real-time PCR and we review the literature as it applies to virus detection in the routine and research laboratory in order to focus on one of the many areas in which the application of real-time PCR has provided significant methodological benefits and improved patient outcomes. However, the technology discussed has been applied to other areas of microbiology as well as studies of gene expression and genetic disease.

BACKGROUND

The polymerase chain reaction (PCR) (1,2) has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties, including virology. The method utilises a pair of synthetic oligonucleotides or primers,

each hybridising to one strand of a double-stranded DNA (dsDNA) target, with the pair spanning a region that will be exponentially reproduced. The hybridised primer acts as a substrate for a DNA polymerase (most commonly derived from the thermophilic bacterium *Thermus aquaticus* and called *Taq*), which creates a complementary strand via sequential addition of deoxynucleotides. The process can be summarised in three steps: (i) dsDNA separation at temperatures >90°C, (ii) primer annealing at 50–75°C, and (iii) optimal extension at 72–78°C (Fig. 1A). The rate of temperature change or ramp rate, the length of the incubation at each temperature and the number of times each set of temperatures (or cycle) is repeated are controlled by a programmable thermal cycler. Current technologies have significantly shortened the ramp times using electronically controlled heating blocks or fan-forced heated air flows to moderate the reaction temperature. Consequently, PCR is displacing some of the gold standard cell culture, antigenaemia and serological assays (3). Existing combinations of PCR and detection assays (called 'conventional PCR' here) have been used to obtain quantitative data with promising results. However, these approaches have suffered from the laborious post-PCR handling steps required to evaluate the amplicon (4).

Traditional detection of amplified DNA relies upon electrophoresis of the nucleic acids in the presence of ethidium bromide and visual or densitometric analysis of the resulting bands after irradiation by ultraviolet light (5). Southern blot detection of amplicon using hybridisation with a labelled oligonucleotide probe is also time consuming and requires multiple PCR product handling steps, further risking a spread of amplicon throughout the laboratory (6). Alternatively, PCR–ELISA may be used to capture amplicon onto a solid phase using biotin or digoxigenin-labelled primers, oligonucleotide probes (oligoprobes) or directly after incorporation of the digoxigenin into the amplicon (7–12). Once captured, the amplicon can be detected using an enzyme-labelled avidin or anti-digoxigenin reporter molecule similar to a standard ELISA format.

The possibility that, in contrast to conventional assays, the detection of amplicon could be visualised as the amplification progressed was a welcome one (13). This approach has provided a great deal of insight into the kinetics of the reaction

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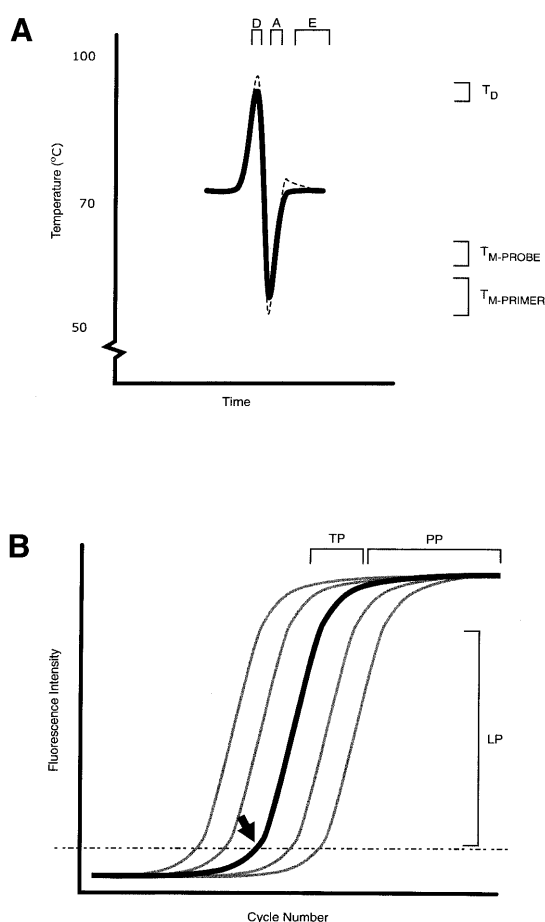


Figure 1. Kinetic amplification. (A) An idealised plot of temperature versus time during a single PCR cycle comprised of the denaturation (D), primer and probe annealing (A) and primer extension (E) steps. At the indicated optimal temperature ranges, dsDNA is denatured (T_D), oligoprobes anneal ($T_{M-PROBE}$) and finally the primers anneal as a precursor to their extension ($T_{M-PRIMER}$). The actual temperature, shown as a dashed line, may overshoot the desired temperature to varying degrees, depending on the quality of the thermocycler employed. (B) The ideal amplification curve of a real-time PCR (bold), when plotted as fluorescence intensity against the cycle number, is a typical sigmoidal growth curve. Early amplification cannot be viewed because the detection signal is indistinguishable from the background. However, when enough amplicon is present, the assay's exponential progress can be monitored as the rate of amplification enters a log-linear phase (LP). Under ideal conditions, the amount of amplicon increases at a rate of approximately one \log_{10} every three cycles. As primers and enzyme become limiting and products inhibitory to the PCR accumulate, the reaction slows, entering a transition phase (TP), eventually reaching the plateau phase (PP) where there is little or no further increase in product yield. The point at which the fluorescence passes from insignificant levels to clearly detectable is called the threshold cycle (C_T ; 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 an optimal titration of template (grey), consisting of higher and lower starting template concentrations, which produce lower or higher C_T values, respectively. Data for the construction of a standard curve are taken from the LP, which subsequently allows the concentration of unknown samples to be determined.

and it is the foundation of kinetic or 'real-time' PCR (Fig. 1B) (6,14–17). Real-time PCR has already proven itself valuable in laboratories around the globe, building on the enormous amount of data generated by conventional PCR assays.

The monitoring of accumulating amplicon in real time has been made possible by the labelling of primers, probes or amplicon with fluorogenic molecules. This chemistry has clear

benefits over radiogenic oligoprobes that include an avoidance of radioactive emissions, ease of disposal and an extended shelf life (18).

The increased speed of real-time PCR is largely due to reduced cycle times, removal of post-PCR detection procedures and the use of fluorogenic labels and sensitive methods of detecting their emissions (19,20). The reduction in amplicon size generally recommended by the creators of commercial real-time assays may also play a role in this speed, however we have shown that decreased product size does not necessarily improve PCR efficiency (21).

The disadvantages of using real-time PCR in comparison with conventional PCR include the inability to monitor amplicon size without opening the system, the incompatibility of some platforms with some fluorogenic chemistries, and the relatively restricted multiplex capabilities of current applications. Also, the start-up expense of real-time PCR may be prohibitive when used in low-throughput laboratories. These shortcomings are mostly due to limitations in the system hardware or the available fluorogenic dyes or 'fluorophores', both of which will be discussed in more detail.

Because most of the popular real-time PCR chemistries depend upon the hybridisation of an oligoprobe to its complementary sequence on one of the strands of the amplicon, the use of more of the primer that creates this strand is beneficial to the generation of an increased fluorescent signal (22). Asymmetric PCR, as this is known, has been shown to produce improved fluorescence from a hairpin oligoprobe PCR (23) and we have found it directly applicable to other oligoprobe-hybridisation assays.

The most commonly used fluorogenic oligoprobes rely upon fluorescence resonance energy transfer (FRET; Fig. 2) 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. FRET is a spectroscopic process by which energy is passed between molecules separated by 10–100 Å that have overlapping emission and absorption spectra (24,25). Förster primarily developed the theory behind this process: the mechanism is a non-radiative induced-dipole interaction (26). The efficiency of energy transfer is proportional to the inverse sixth power of the distance (R) between the donor and acceptor ($1/R^6$) fluorophores (27,28).

Post-amplification manipulation of the amplicon is not required for real-time PCR, therefore these assays are described as 'closed' or homogeneous systems. The advantages of homogeneous systems include a reduced result turnaround, minimisation of the potential for carry-over contamination and the ability to closely scrutinise the assay's performance (29). In addition, real-time PCR has proven cost effective when implemented in a high throughput laboratory (30), particularly when replacing conventional, culture-based approaches to virus detection.

In the remainder of this article, the theory behind real-time PCR will be reviewed and its rapidly increasing use in the fields of human virology will be used as an illustration.

AMPLICON DETECTION

In the following section we will focus on the detection processes that discriminate real-time PCR from conventional

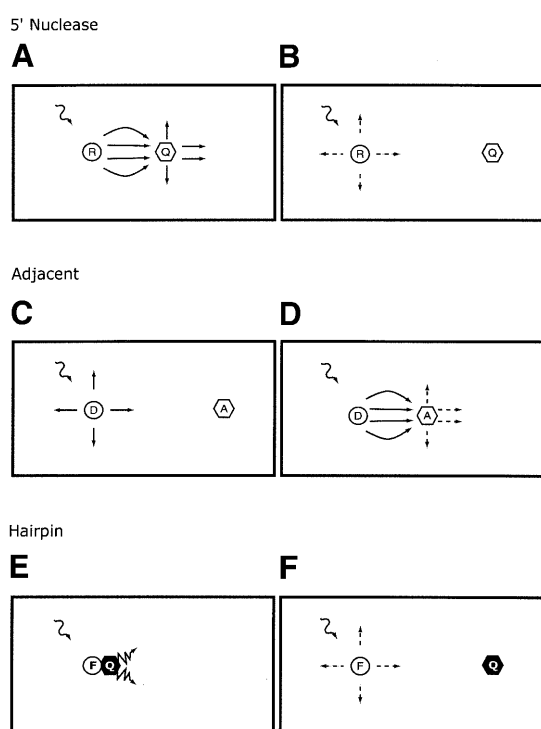


Figure 2. Fluorogenic mechanisms. When a 5' nuclease probe's reporter (R) and quencher (Q, open) are in close proximity as in (A), the quencher 'hijacks' the emissions that have resulted from excitation of the reporter by the instrument's light source. The quencher then emits this energy (solid arrows). When the fluorophores are separated beyond a certain distance, as occurs upon hydrolysis as depicted in (B), the quencher no longer exerts any influence and the reporter emits at a distinctive wavelength (dashed arrows) which is recorded by the instrument. In the reverse process as depicted in (C) using adjacent oligoprobes, the fluorophores begin as separated entities. A signal from the acceptor (A) can only be generated when the donor (D) comes into close proximity as shown in (D). This occurs as the result of adjacent hybridisation of the oligoprobes to the target amplicon. In (E) another form of quenching is shown, caused by the intimate contact of labels attached to hairpin oligoprobes (molecular beacon, sunrise or scorpion). The fluorophore (F) and a NFQ (Q, closed) interact more by collision than FRET, disrupting each other's electronic structure and directly passing on the excitation energy which is dissipated as heat (jagged, dashed arrows). When the labels are separated by disruption of the hairpin, as is the case in (F), the fluorophore is free to fluoresce (dashed arrows).

PCR assays. There are five major chemistries currently in use, and they can be classified into amplicon sequence specific or non-specific methods of real-time PCR detection (31). Each of the chemistries has an associated nomenclature to describe the fluorescent labels; however, for general discussion, fluorophore will continue to be used to describe these moieties. Although this review focuses on the use of these chemistries in real-time applications, they can also be used as a label for end-point amplicon detection.

DNA-binding fluorophores

The basis of the sequence non-specific detection methods is the DNA-binding fluorogenic molecule. Included in this group are the earliest and simplest approaches to real-time PCR. Ethidium bromide (32), YO-PRO-1 (33,34) and SYBR[®] green 1 (35) all fluoresce when associated with dsDNA which is exposed to a suitable wavelength of light. This approach

requires less specialist knowledge than the design of fluorogenic oligoprobes, is less expensive and does not suffer when the template sequence varies, which may abrogate hybridisation of an oligoprobe (36). Formation of primer-dimer (37) is common and, together with the formation of specific products, is strongly associated with entry of the PCR into the plateau phase (Fig. 1B) (38,39). Association of a DNA-binding fluorophore with primer-dimer or other non-specific amplification products can confuse interpretation of the results. Adding a short, higher temperature incubation after the extension step in which fluorescence data are acquired minimises the contribution of these products to the fluorescence signal (40). The problem of primer-dimer can also be addressed using software capable of fluorescent melting curve analysis. This method makes use of the temperature at which the dsDNA amplicon is denatured (T_D ; Fig. 1A). The shorter primer-dimer can be discriminated by its reduced T_D compared with the full-length amplicon. Analysis of the melting curves of amplicon in the presence of SYBR green 1 has demonstrated that the practical sensitivity of DNA-binding fluorophores is limited by non-specific amplification at low initial template concentrations.

DNA binding fluorophores also increase the T_D and broaden the melting transition, requiring substantial sequence change to produce a shift in the T_D . Oligoprobes are able to discriminate single point mutations using the temperature at which 50% of oligoprobe-target duplexes separate (41). This temperature is called the melting temperature (T_M) and it is dependent upon the concentration of the dsDNA, its length, nucleotide sequence and the solvent composition, and is often confused with T_D (Fig. 1A) (42).

Linear oligoprobes

The use of a pair of adjacent, fluorogenic hybridisation oligoprobes was first described in the late 1980s (43,44) and, now known as 'HybProbes', they have become the method of choice for the LightCycler[™] (Roche Molecular Biochemicals, Germany), a capillary-based, microvolume fluorimeter and thermocycler with rapid temperature control (20,45). The upstream oligoprobe is labelled with a 3' donor fluorophore (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 nt of each other, sometimes attracting the name 'kissing' probes (Figs 2C, D and 3C). The plastic and glass composite capillaries are optically clear and act as cuvettes for fluorescence analysis, as well as facilitating rapid heat transfer. Capillaries are rotated past a blue light-emitting diode and fluorescence is monitored by three photodetection diodes with different wavelength filters. The temperature is varied by rapidly heating and cooling air using a heating element and fan which produce ramp rates of 20°C/s, prolonging polymerase survival (46). Additionally, because the oligoprobes are not significantly hydrolysed during amplification (47) and the LightCycler is able to monitor the changes in fluorescence emission during denaturation of the adjacent oligoprobes from their amplicon, this system can perform single tube genotyping. This capability, which makes use of fluorescent melting curve analysis, provides significant information about the sequence to which the oligoprobes are binding. Mutation(s) under one or both oligoprobes can be determined by the decrease in melting

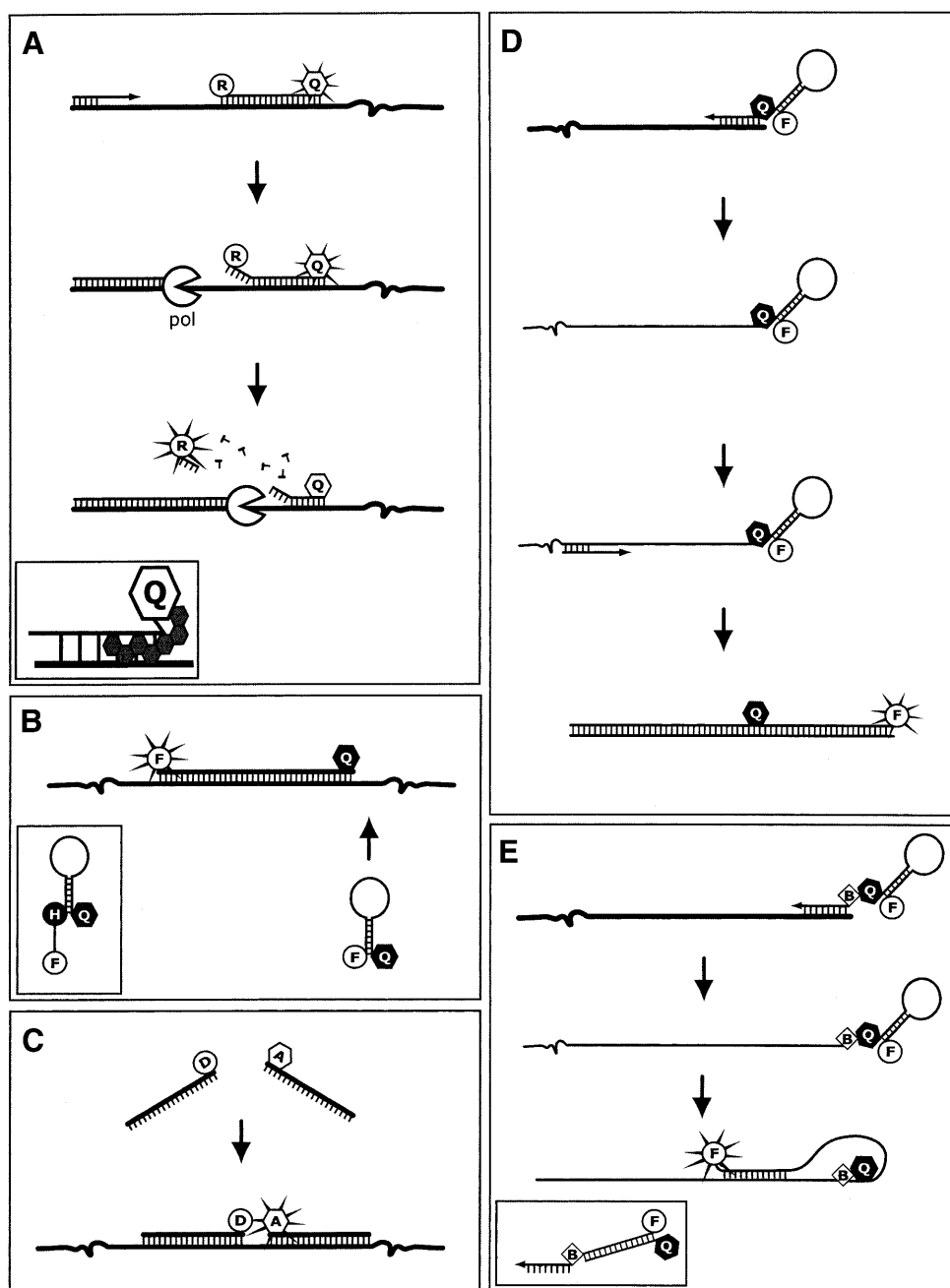


Figure 3. Oligoprobe chemistries. (A) 5' Nuclease oligoprobes. As the DNA polymerase (pol) progresses along the relevant strand, it displaces and then hydrolyses the oligoprobe via its 5'→3' endonuclease activity. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q, open), it is able to release excitation energy at a wavelength that is monitored by the instrument and different from the emissions of the quencher. Inset shows the NFQ and MGB molecule that make up the improved MGB nuclease-oligoprobes. (B) Hairpin oligoprobes. Hybridisation of the oligoprobe to the target separates the fluorophore (F) and non-fluorescent quencher (Q, closed) sufficiently to allow emission from the excited fluorophore, which is monitored. Inset shows a wavelength-shifting hairpin oligoprobe incorporating a harvester molecule. (C) Adjacent oligoprobes. Adjacent hybridisation results in a FRET signal due to interaction between the donor (D) and acceptor (A) fluorophores. This bimolecular system acquires its data from the acceptor's emissions in an opposite manner to the function of nuclease oligoprobe chemistry. (D) Sunrise primers. The opposite strand is duplicated so that the primer's hairpin structure can be disrupted. This separates the labels, eliminating the quenching in a similar manner to the hairpin oligoprobe. (E) Scorpion primers. The primer does not require extension of the complementary strand; in fact it blocks extension to ensure that the hairpin in the probe is only disrupted by specific hybridisation with a complementary sequence designed to occur downstream of its own, nascent strand. Inset shows a duplex scorpion that exchanges the stem-loop structure for a primer element terminally labelled with the fluorophore and a separate complementary oligonucleotide labelled with a quencher at the 5' terminus.

temperature that they incur due to destabilisation of the oligoprobe/target duplex (Fig. 4). This has imparted significant improvements in speed upon the diagnosis of genetic disease as well as a growing number of multiplex PCR approaches for

the detection of related viral pathogens. Despite the fact that the hybridisation does not reach equilibrium using these ramp rates, the apparent T_M values are both reproducible and characteristic of a given probe/target duplex (48). However,

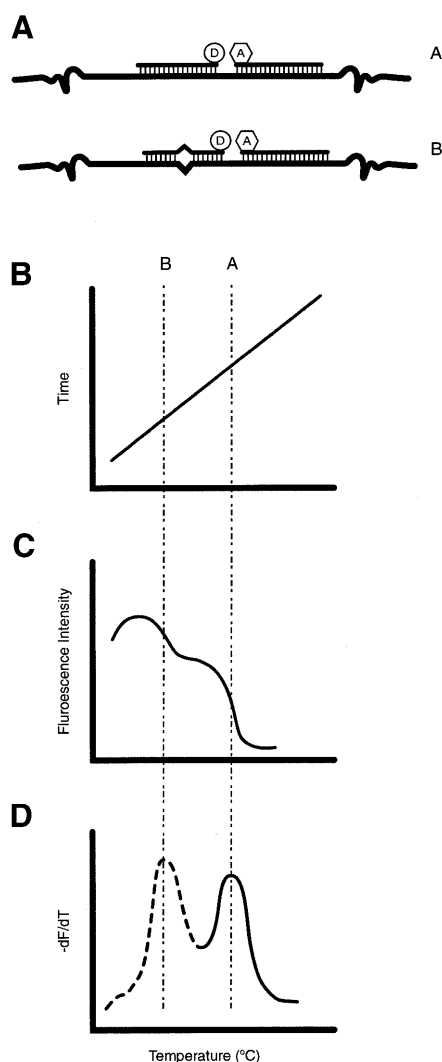


Figure 4. Fluorescent melting curve analysis. At the completion of a real-time PCR using a pair of adjacent oligoprobes, the reaction can be cooled to a temperature below the expected T_M of the oligoprobes then heated above 85°C at a fraction of a degree per second (B). During heating, the emissions of the acceptor can be constantly acquired (C). Software calculates the negative derivative of the fluorescence over time, producing clear peaks that indicate the T_M of the oligoprobe-target melting transition (D). When one or more mutations are present under one or both oligoprobes (A), this T_M is shifted and this shift can be used diagnostically to discriminate single nucleotide polymorphisms in the template. Ideally, one of the oligoprobes, the anchor, is designed to bind to a stable sequence region, whereas the other, sensor, will span the mismatch. Mismatches near the centre of the probe and flanked by G:C pairs are more destabilising than mismatches near the ends of the oligoprobe flanked by A:T pairs.

the capillaries are fragile and require some experience to handle (49).

When comparing signals from the different chemistries, the destruction of nuclease oligoprobes continues despite a plateau in product accumulation whereas SYBR green 1 fluorescence in the no template control generally increases non-specifically during later cycles. Adjacent oligoprobe fluorescence begins to decrease as the rate of collision between the growing numbers of complementary amplicon strands increases favouring the formation of dsDNA over the hybridisation of oligoprobe to its target DNA strand. Additionally, there is the possibility that

some oligoprobe is consumed by sequence-related endonuclease activity (50,51). All three oligoprobe chemistries (SYBR Green I, nuclease and adjacent oligoprobes) seem capable of detecting amplified product with approximately the same sensitivity (20).

Combinations of the above approaches are now appearing as more users of the instrumentation become familiar with the concepts behind real-time PCR and contribute to the literature. If a sequence-specific, fluorophore-labelled linear oligoprobe is added to a SYBR green 1 mix, currently called the Bi-probe system, FRET will occur and an additional layer of specificity can be obtained (44,52,53). An assay using a BODIPY[®]FL-labelled oligoprobe was adapted to run in the LightCycler using a β -globin target sequence (54). The probe was designed so that the fluorophore was located on a terminal cytosine and was quenched by proximity with a complementary guanine. The assay demonstrated that quenching varies linearly with the concentration of template across a defined concentration range. The commonly used fluorophore FITC is inherently quenched by deoxyguanosine nucleotides. The level of quenching can be increased if more guanines are present or a single guanine is located in the first overhang position, 1 nt beyond the fluorophore-labelled terminus of the probe. This approach to amplicon detection is easier to design than fluorogenic oligoprobes, simpler to synthesise and use in real-time PCR and does not require a DNA polymerase with nuclease activity (55).

The light-up probe is a peptide nucleic acid to which the asymmetric cyanine fluorophore thiazole orange is attached (56). When hybridised with a nucleic acid target, either as a duplex or triplex, depending on the oligoprobe's sequence, the fluorophore becomes strongly fluorescent. These probes do not interfere with the PCR, do not require conformational change, are sensitive to single nucleotide mismatches allowing fluorescence melting analysis, and because a single reporter is used, a direct measurement of fluorescence can be made instead of the measurement of a change in fluorescence between two fluorophores (56,57). However, non-specific fluorescence has been reported during later cycles using these probes (58).

5' Nuclease oligoprobes

In the late 1980s homogeneous assays were few and far between, but rapid advances in thermocycler instrumentation and the chemistry of nucleic acid manipulation have since made these assays commonplace. The success of these assays revolves around a signal changing in some rapid and measurable way upon hybridisation of a probe to its target (59). By using an excess, the time required for hybridisation of an oligoprobe to its target, especially when the amount of that target has been increased by PCR or some other amplifying process, is significantly reduced (41,59).

In 1991, Holland *et al.* (6) described a technique that was to form the foundation for homogeneous PCR using fluorogenic oligoprobes. Amplicon was detected by monitoring the effect of *Taq* DNA polymerase's 5'→3' endonuclease activity on specific oligoprobe/target DNA duplexes. The radiolabelled products were examined using thin layer chromatography and the presence or absence of hydrolysis was used as an indicator of duplex formation. These oligoprobes contained a 3' phosphate moiety, which blocked their extension by the

polymerase, but otherwise had no effect on the amplicon's yield.

The desirable criteria for an oligoprobe label are (i) easy attachment of the label to DNA, (ii) detectability at low concentrations, (iii) detectability using simple instrumentation, (iv) production of an altered signal upon specific hybridisation, (v) biological safety, (vi) stability at elevated temperatures and (vii) an absence of interference with the activity of the polymerase (6,18).

An innovative approach used nick-translation PCR in combination with dual-fluorophore labelled oligoprobes (14). In the first truly homogenous assay of its kind, one fluorophore was added to the 5' terminus and one to the middle of a sequence specific oligonucleotide probe. When in such close proximity, the 5' reporter fluorophore (6-carboxy-fluorescein) transferred laser-induced excitation energy by FRET to the 3' quencher fluorophore (6-carboxy-tetramethyl-rhodamine; TAMRA), which reduced the lifetime of the reporter's excited state by taking its excess energy and emitting it as a fluorescent signal of its own (Fig. 2A and B). TAMRA emitted the new energy at a wavelength that was monitored but not utilised in the presentation of data. However, when the oligoprobe hybridised to its template, the fluorophores were released due to hydrolysis of the oligoprobe component of the probe/target duplex. Once the labels were separated, the reporter's emissions were no longer quenched and the instrument monitored the resulting fluorescence. These oligoprobes have been called 5' nuclease, hydrolysis or TaqMan[®] oligoprobes (Fig. 3A). Nuclease oligoprobes have design requirements that are applicable to the other linear oligoprobe chemistries, including (i) a length of 20–40 nt, (ii) a GC content of 40–60%, (iii) no runs of a single nucleotide, particularly G, (iv) no repeated sequence motifs, (v) an absence of hybridisation or overlap with the forward or reverse primers and (vi) a T_M at least 5°C higher than that of the primers, to ensure the oligoprobe has bound to the template before extension of the primers can occur (60).

This technology, however, required the development of a platform to excite and detect fluorescence as well as perform thermal cycling. A charge-coupled device had been described in 1992 for the quantification of conventional reverse transcription (RT)-PCR products (61). In 1993 this approach was combined with a thermal cycler resulting in the first real-time PCR fluorescence excitation and detection platform (29). To date, the ABI Prism[®] 7700 sequence detection system (Perkin Elmer Corporation/Applied Biosystems, USA) has been the main instrument used for 5' nuclease oligoprobes. Non-PCR related fluorescence fluctuations have been normalised using 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 reporter signal 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 (62).

The fractional cycle number at which the real-time fluorescence signal mirrors progression of the reaction above the background noise was used as an indicator of successful target amplification (63). This threshold cycle (C_T) is defined as the PCR cycle in which the gain in fluorescence generated

by the accumulating amplicon exceeds 10 standard deviations of the mean baseline fluorescence, using data taken from cycles 3 to 15 (Fig. 1B) (64). The C_T is proportional to the number of target copies present in the sample (17).

A recent improvement to the nuclease oligoprobe has resulted in the minor groove binding (MGB) oligoprobes (Fig. 3A, inset). This chemistry replaces the standard TAMRA quencher with an NFQ and incorporates a molecule that stabilises the oligoprobe-target duplex by folding into the minor groove of the dsDNA (65). This allows the use of very short (14 nt) oligoprobes, which are ideal for detecting single nucleotide polymorphisms (SNPs). A related use of dual labelled oligonucleotide sequences has been to provide the signal-generating portion of the DzyNA-PCR system (66). Here, the reporter and quencher are separated after cleavage of the probe by a DNAzyme, which is created during PCR as the complement of an antisense DNAzyme sequence included in the 5' tail of one of the primers. Upon cleavage, the dual labelled substrate releases the fluorophores and generates a signal in an analogous manner to the 5' nuclease probe.

Hairpin oligoprobes

Molecular beacons were the first hairpin oligoprobes and are a variation of the dual-labelled nuclease oligoprobe (Fig. 3B). The hairpin oligoprobe's fluorogenic labels are called fluorophore and quencher, and they 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 which results in quenching either by FRET or a direct energy transfer by a collisional mechanism due to the intimate proximity of the labels (Fig. 2E and F) (67). 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 and fluorescent emissions are monitored during each cycle (68). The occurrence of a mismatch between a hairpin oligoprobe 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 alternate conformation. Therefore, hairpin oligoprobes have been shown to be more specific than the more common linear oligoprobes making them ideal candidates for detecting SNPs (67). The quencher, 4-(4'-dimethylamino-phenylazo)-benzene (DABCYL), differs from that described for the nuclease oligoprobes because it is an NFQ.

The wavelength-shifting hairpin probe is a recent improvement to this chemistry which makes use of a second, harvesting fluorophore. The harvester passes excitation energy acquired from a blue light source and releases it as fluorescent energy in the far-red wavelengths. The energy can then be used by a receptive 'emitter' fluorophore that produces light at characteristic wavelengths (Fig. 3B, inset). This offers the potential for improved multiplex real-time PCR and SNP analysis (Fig. 4), using currently available instruments (69). Because the function of these oligoprobes depends upon correct hybridisation of the stem, accurate design is crucial to their function (47).

Self-fluorescing amplicon

The self-priming amplicon is similar in concept to the hairpin oligoprobe, except that the label becomes irreversibly incorporated into the PCR product (Fig. 3D and E). Two approaches have been described: sunrise primers (now commercially called Amplifluor™ hairpin primers) and scorpion primers (31,70). The sunrise primer consists of a 5' fluorophore and a DABCYL NFAQ. The labels are separated by complementary stretches of sequence that create a stem when the sunrise primer is closed. At the 3' terminus is a target-specific primer sequence. The sunrise primer's sequence is intended to be duplicated by the nascent complementary strand and, in this way, the stem is destabilised, the two fluorophores are held ~20 nt (70 Å) apart and the fluorophore is free to emit its excitation energy for monitoring (70). This system could suffer from non-specific fluorescence due to duplication of the sunrise primer sequence during the formation of primer-dimer.

The scorpion primer is almost identical in design except for an adjacent hexethylene glycol molecule that blocks duplication of the signalling portion of the scorpion. In addition to the difference in structure, the function of scorpion primers differs slightly in that the 5' region of the oligonucleotide is designed to hybridise to a complementary region within the amplicon. This hybridisation forces the labels apart disrupting the hairpin and permitting emission in the same way as hairpin probes (31).

VIRAL QUANTITATION

The majority of diagnostic PCR assays reported to date have been used in a qualitative, or 'yes/no' format. The development of real-time PCR has brought true quantitation of target nucleic acids out of the pure research laboratory and into the diagnostic laboratory.

Determining the amount of template by PCR can be performed in two ways: as relative quantitation and as absolute quantitation. Relative quantitation describes changes in the amount of a target sequence compared with its level in a related matrix. Absolute quantitation states the exact number of nucleic acid targets present in the sample in relation to a specific unit (71). Generally, relative quantitation provides sufficient information and is simpler to develop. However, when monitoring the progress of an infection, absolute quantitation is useful in order to express the results in units that are common to both scientists and clinicians and across different platforms. Absolute quantitation may also be necessary when there is a lack of sequential specimens to demonstrate changes in virus levels, no suitably standardised reference reagent or when the viral load is used to differentiate active versus persistent infection.

A very accurate approach to absolute quantitation by PCR is the use of competitive co-amplification of an internal control nucleic acid of known concentration and a wild-type target nucleic acid of unknown concentration, with the former designed or chosen to amplify with an equal efficiency to the latter (72–76). However, while conventional competitive PCR is relatively inexpensive, real-time PCR is far more convenient, reliable and better suited to quick decision making in a clinical situation (77,78). This is because conventional, quantitative, competitive PCR (qcPCR) requires significant development and optimisation to ensure reproducible performance and a

predetermined dynamic range for both the amplification and detection components (79).

Although a comparison of absolute standard curves, relative standard curves and C_T values produces similar final values (80), the general belief remains that an internal control in combination with replicates of each sample are essential for reliable quantitation by PCR (38,39). Unfortunately, real-time PCR software with the ability to calculate the concentration of an unknown by comparing signals generated by an amplified target and internal control is only beginning to emerge. This issue will hopefully be addressed in upcoming commercial releases (81). Therefore, the next best approach to quantitation by PCR is the use of an external standard curve. This approach relies upon titration of an identically amplified template, in a related sample matrix, within the same experimental run. While the external standard curve is the more commonly described approach, it suffers from uncontrolled and unmonitored inter-tube variations. Because of this omission, such experiments should be described as semi-quantitative. Despite this sub-optimal approach, fluorescence data is generally collected from PCR cycles that span the linear amplification portion of the reaction where the fluorescent signal and the accumulating DNA are proportional. Because the emissions from fluorescent chemistries are temperature dependent, data is generally acquired only once per cycle at the same temperature in order to monitor amplicon yield (45). The C_T of the sample at a specific fluorescence value can then be compared with similar data collected from a series of standards by the calculation of a standard curve. The determination of the C_T depends upon the sensitivity and ability of the instrument to discriminate specific fluorescence from background noise, the concentration and nature of the fluorescence-generating component and the amount of template initially present.

Real-time PCR offers significant improvements to the quantitation of viral load because of its enormous dynamic range that can accommodate at least eight \log_{10} copies of nucleic acid template (33,52,77,82–89). This is made possible because the data are chosen from the linear phase (LP; Fig. 1B) of amplification where conditions are optimal, rather than the end-point where the final amount of amplicon present may have been affected by inhibitors, poorly optimised reaction conditions or saturation by inhibitory PCR by-products and double-stranded amplicon. The result of taking data from the end-point is that there may not be a relationship between the initial template and final amplicon concentrations.

Real-time PCR is also an attractive alternative to conventional PCR for the study of viral load because of its low inter-assay and intra-assay variability (77,87,90) and its equivalent or greater analytical sensitivity in comparison with traditional viral culture, or conventional single-round, and nested PCR (77,85,91–96). Real-time PCR has been reported to be at least as sensitive as Southern blot (92). However, these reports could be an over-estimate due to the choice of smaller targets, which amplify more efficiently, or due to the use of different or improved primers for the real-time assays because the use of software to design optimised primers and oligoprobes is more common.

When this increased sensitivity and broad dynamic range are combined, it is possible to quantitate template from samples containing a large range of concentrations, as is often the case in patient samples. This avoids the need for dilution of the

amplicon prior to conventional detection or repeat of the assay using a diluted sample because the first test result falls outside the limits of the assay. These are problems encountered when using some conventional qPCR assay kits, which cannot encompass high viral loads whilst maintaining suitable sensitivity (52,97–99). The flexibility of real-time PCR is also demonstrated by its ability to detect one target in the presence of a vast excess of another target in duplexed assays (84).

Viral load is also a useful indicator of the extent of active infection, virus–host interactions and the response to antiviral therapy, all of which can play a role in the treatment regimen selected (100,101). Conventional quantitative PCR has already proven the benefits of applying nucleic acid amplification to the monitoring of viral load as a useful marker of disease progression and as a component of studies into the efficacy of antiviral compounds (74,100,102–104). The severity of some diseases has been shown to correlate with the viral load making real-time PCR quantitation useful to study not simply the presence of a virus but the role of viral reactivation or persistence in the progression of disease (78,82,91,105–112).

An example of the benefits which real-time PCR has brought to the quantitative detection of human cytomegalovirus (CMV) is seen in patients who are immunosuppressed following solid organ or bone marrow transplantation. Although qualitative detection of CMV DNA by PCR has been used as an indicator for the success of antiviral therapy, quantitative assays are preferred in order to monitor patient's therapeutic responses. Moreover, since it has been postulated that the monitoring of viral replication over time is a more reliable indicator of a developing viral disease than the determination of absolute viral amounts at a single point of time, several quantitative assays have been established and evaluated to increase diagnostic accuracy. Quantitative competitive assays based on end-point analysis have displayed detection limits of 5×10^1 genome equivalents (ge) per assay and a dynamic range of 5×10^1 – 5×10^4 ge/assay (113). Hybridisation-based assays covered approximately the same dynamic range of four orders of magnitude with detection limits of 20 ge/assay (114,115). Although these assays possess dynamic ranges that may be sufficient for most clinical applications, they display a high inter- and intra-assay variability, up to 40% (115).

In contrast, one of the first published real-time PCR assays for the detection of CMV DNA could be performed in <90 min, spanned a dynamic range of six to seven orders of magnitude with a detection limit of at least 10 ge/assay and an inter-assay and intra-assay variability of <10% and <5%, respectively, using plasma samples from bone marrow transplant patients (116).

MULTIPLEX REAL-TIME PCR

Multiplexing (using multiple primers to allow amplification of multiple templates within a single reaction) is a useful application of conventional PCR (117). However, its transfer to real-time PCR has confused its traditional terminology. The term multiplex real-time PCR is more commonly used to describe the use of multiple fluorogenic oligoprobes for the discrimination of multiple amplicons. The transfer of this technique has proven problematic because of the limited number of fluorophores available (14) and the common use of a monochromatic energising light source. Although excitation by a single

wavelength produces bright emissions from a suitably selected fluorophore, this restricts the number of fluorophores that can be included (69). Recent improvements to the design of the hairpin primers, and hairpin and nuclease oligoprobes as well as novel combinations of fluorophores such as in the bi-probe and light-up probe systems, have promised the ability to discriminate an increasing number of targets.

The discovery and application of the non-fluorescent quenchers has liberated some wavelengths that were previously occupied by the emissions from the early quenchers themselves. This breakthrough has permitted the inclusion of a greater number of spectrally discernable oligoprobes per reaction, and highlighted the need for a single non-fluorescent quencher, which can quench a broad range of emission wavelengths (e.g. 400–600 nm). Early real-time PCR systems contained optimised filters 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 when compared with the discriminatory abilities of conventional multiplex PCR. More recent real-time PCR platforms have incorporated either multiple light-emitting diodes to span the entire visible spectrum, or a tungsten light source, which emits light over a broad range of wavelengths. When these platforms incorporate high quality optical filters it is possible to apply any current real-time PCR detection chemistries on the one machine. Nonetheless, these improvements generally allow only four-colour oligoprobe multiplexing, of which one colour is ideally set aside for an internal control to monitor inhibition and perhaps even act as a co-amplified competitor. Some real-time PCR designs have made use of single or multiple nucleotide changes between similar templates to allow their differentiation by T_M (Fig. 4) thus avoiding the need for multiple fluorophores (49,91,118–122). This approach has been used far more commonly in the detection of human genetic diseases where as many as 27 possible nucleotide substitutions have been detected using only one or two fluorophores (48,123–128).

To date, there have only been a handful of truly multiplexed real-time PCR assays described in the literature and few of these have been applied to the diagnosis of infectious disease. Some of these approaches cannot, technically, be considered real-time, homogenous assays because they require interruption of the procedure to transfer template, their fluorescence is detected by end-point analysis or the assays are not performed within the same tube. One of the best viral, multiplex, real-time PCR protocols can discriminate between four retroviral target sequences (129), however, conventional multiplex PCR using end-point detection can easily discriminate more than five different amplified sequences, indicating a greater flexibility when compared with real-time PCR (130–133).

Despite these limitations, a number of assays have been applied to the detection of several viral genomes at once, including the use of non-specific label, SYBR green 1 to detect herpes simplex viruses (HSV), varicella zoster virus (VZV) or CMV, in separate tubes (134) or, by adaptation of a conventional multiplex PCR, to identify HSV-1 and HSV-2, VZV and enteroviruses within a single capillary by applying fluorescent melting curve analysis (121). Another single-tube multiplexed nuclease oligoprobe RT-PCR was capable of simultaneously detecting Influenza A and B in patient's respiratory samples

with improved sensitivity compared with conventional or shell-viral culture (95).

Future developments of novel chemistries such as combinatorial fluorescence energy transfer tags (135), and improvements to the design of real-time instrumentation and software will greatly enhance the future of multiplex real-time PCR.

APPLICATIONS TO VIROLOGY

Real-time PCR has been extremely useful for studying viral agents of infectious disease and helping to clarify disputed infectious disease processes. Most of the assays presented in the literature allow an increased frequency of virus detection compared with conventional techniques, which makes the implementation of real-time PCR attractive to many areas of virology.

Of course, real-time PCR has proven increasingly valuable for general virological studies, although increasingly, these applications are difficult to review due to the nature of their use as a tool rather than the focus of published study. Such studies have investigated the role of viruses in a range of diseases by simply confirming the presence or absence of the virus (136,137) or, in the future, by monitoring the levels of specific gene activity (138) as a result of growth under manipulated conditions. Altered viral entry or replication, caused by the modification of target tissues, can also be followed using real-time PCR as can links between virus replication and the expression of cellular genes (139–141).

Real-time PCR has enhanced the speed and scope of measuring viral strain and titre differences in patients displaying different syndromes due to varieties of the same virus (106). Also, epidemiological studies have been improved in speed and scope through the use of real-time PCR because it can reliably measure the amount of two nucleic acid targets within a single reaction (91,142). New chemistries have allowed better discrimination of multiple viral genotypes within a single reaction vessel (143) and provided an alternative to methods of virus detection based on morbidity and mortality assays.

The use of real-time PCR has provided insight into the role(s) some compounds have on PCR inhibition as well as shedding light on the efficiency of different nucleic acid extraction methods, from a diverse range of sample types (144–146). This ability to utilise template from a range of sample types fulfils a requirement for an ideal detection system, which is the ability to apply a single technology across many fields. This flexibility is highlighted by the detection of viral nucleic acids derived, in different ways, from plants (147–149), animals (86,89,94,150–152), urban sludge (85,153), tissue culture (23,77,96,154–160), various solid tissues (108,118,161–166), cerebrospinal fluid (49,167,168), peripheral blood mononuclear cells (82,93,105,112,169–171), plasma (81,88,90,172–176), serum (30,33,36,87,97,99,109,177–181), swabs (182,183), saliva (106) and urine (78,122,184,185). Also, chronic conditions such as sarcoma (186–189), carcinoma (92,111), cervical intraepithelial neoplasia (190–192), and lymphoproliferative disorders (193,194) can be relatively easily studied to investigate direct or indirect links with viral infection. These studies have targeted viruses which include the flaviviruses (33,36,96,109,195), hepadnaviridae (52,97), herpesviruses (21,77,78,82–84,91,92,98,105,106,108,

111,112,144,167,171), orthomyxoviruses (95), parvoviruses (88), papovaviruses (122,143,145), paramyxoviruses (94), picornaviruses (85,86), retroviruses (90,93,156,196,197) and TT virus (137). Viral load monitoring by real-time PCR has also proven beneficial for studying patients following organ transplant (21,83,107,198–201).

This technology has now become an essential tool in the thorough assessment of viral gene therapy vectors prior to their use in clinical trials. Nuclease oligoprobes have been most commonly reported for these studies, which assess the biodistribution, function and purity of these drug preparations (164,197,202–205).

Additionally, the study of emerging viruses has been complemented by the use of real-time PCR as a tool to demonstrate links between unique viral sequences and patient clinical signs and symptoms (94,96,150,160,206,207).

The speed and flexibility of real-time PCR has also proven useful to commercial interests for the screening of microbial contamination of large-scale reagent preparations produced from eukaryotic expression systems (208,209).

CONCLUSIONS AND SUMMARY

Advances in the development of fluorophores, nucleotide labelling chemistries, and the novel applications of oligoprobe hybridisation have provided real-time PCR technology with a broad enough base to ensure its acceptance. Recently, instrumentation has appeared that is capable of incredibly short cycling times combined with the ability to detect and differentiate multiple amplicons. New instruments are also flexible enough to allow the use of any of the chemistries described in this review making real-time nucleic acid amplification an increasingly attractive and viable proposition for the routine diagnostic laboratory. In many cases these laboratories perform tissue culture to isolate virus and serological methods to confirm the identity of the isolate, which may take a considerable, and clinically relevant, amount of time.

The familiarity that leads to comfortable routine use of a technology is now apparent in the inclusion of the fluorogenic oligoprobe chemistries in many laboratories. According to the literature, the most widely used format is the 5' endonuclease oligoprobe although that is most likely due to its commercial maturity. The more recently developed oligoprobe chemistries have been used in a number of innovative applications and it is apparent from the rate and content of real-time PCR-related publications that they are becoming more widely accepted.

Recent developments in multiplex real-time PCR have suggested a future in which easy identification, genotyping and quantitation of viral targets in single, rapid reactions will be commonplace. Of course, this technology is by no means restricted to virology, as significant achievements have appeared in the area of mutation detection, applying all the benefits described above to enhance the detection of genetic disease and, where applicable, allow quantitation of the extent of such genetic changes.

Micro- and macro-array technology will surely play some chimeric role in the real-time PCR of future research, but for now there is significant potential for routine, research and commercial interests to redesign existing systems for greater sophistication, flexibility and the ability to generate high quality quantitative data. The development of assays capable

of real-time PCR that can discriminate as many targets as conventional multiplex PCR assays, whilst producing quantitative data at a greatly increased speed, will consolidate fluorogenic nucleic acid amplification as a routine tool for the laboratory of tomorrow.

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