10 Real-time Fluorescent PCR Techniques to Study Microbial–Host Interactions

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CONTENTS

◆◆◆◆◆◆ INTRODUCTION

The development and introduction of new investigative molecular technologies have begun to change the way we think about virulence and pathogenicity. One such technology is the real-time polymerase chain reaction (PCR). In the context of infectious disease, diagnostic microbiology can be divided into two broad sections: routine qualitative screening or detailed qualitative and quantitative investigations of the interplay between the microbe and host. The following review will describe how real-time PCR performs and how it may be used to detect microbial pathogens and elucidate the relationship they form with their host.

Research and diagnostic microbiology laboratories contain a mix of traditional and leading edge, in-house and commercial assays for the detection of microbes and the effects they impart upon target tissues, organs and systems. The wide variety of assays is the result of many factors including the perceived reliability of familiar techniques and

technologies, the scope of a test to perform the required task, the existence and cost of commercial tests able to detect microbes of interest, the experience a laboratory has acquired with a particular technique and finally the degree of support offered by the assay manufacturer. Additionally, the cost of a new system may be over-estimated prior to its use in the laboratory, adding to slow uptake of new technologies. In the modern microbiology laboratory, an increasing role is played by molecular techniques and, in particular, the PCR (Freymuth *et al.*, 1995; Mullis and Faloona, 1987). The PCR has undergone significant change over the last decade, to the extent that only a small proportion of scientists have been able or willing to keep abreast of the latest offerings. This chapter will review these changes, bringing the reader up to date with the second-generation of PCR technology: kinetic or real-time PCR, a tool gaining widespread acceptance in many scientific disciplines but especially in the microbiology laboratory (Ginzinger, 2002; Whelan and Persing, 1996).

◆◆◆◆◆◆ THE ABC OF PCR

Microbiology is in the midst of a new era. Technologies that detect nucleic acids have driven the evolution of a plethora of new experimental tools rapidly replacing some traditional methods, which depend on microbial phenotype rather than genotype. For example, it has become common to relegate viral culture to specialised virology laboratories rather than include it in the routine diagnostic laboratory, which can then focus on rapid result turnaround (Carman, 2001). The speed with which a negative result is provided is often as important as the return of a positive result and, when cultures can require weeks to complete, the PCR offers an attractive alternative (Carman, 2001). Of course, speed is the result of a number of factors, and they must all be taken into account when assessing the benefits of any new PCR-based assay. Faster assays must be described using the final optimised and validated test performed on real-world specimens instead of presenting preliminary data using idealised templates and conditions. Unfortunately, incomplete PCR assays are frequently rushed into publication resulting in problems reproducing data in other laboratories.

The PCR is the most commonly used molecular technique to detect and study microbes, appealing more widely than specific commercial template amplification technologies such as Abbott's ligase chain reaction (LCR) (Barany, 1991), Organon Teknika's nucleic acid sequence-based amplification (NASBA) (Compton, 1991; Kievits *et al.*, 1991), Becton Dickinson's strand-displacement amplification (SDA) (Walker *et al.*, 1992) and Gen-Probe's transcription-mediated amplification (TMA). PCR has also been applied more widely than the signal amplification methods such as Bayer's branched DNA (bDNA) and Digene's Hybrid Capture (Fredricks and Relman, 1999; Whelan and Persing, 1996; Persing, 1993). The PCR method utilises a pair of synthetic oligonucleotides called

primers, each one hybridising in a 5′ to 3′ orientation to a single strand of a DNA target. The primer pair spans a region that is exponentially duplicated during the subsequent reaction. Each hybridised primer forms a starting point for the production of complementary DNA via the action of a DNA polymerase derived from thermophilic bacteria. The process of primer extension creates a strand that is the complement of the template through the sequential addition of deoxynucleotides. The PCR can be summarised in three steps: (i) double-stranded DNA (dsDNA) separation at temperatures above 90°C, (ii) primer annealing at a specific temperature commonly between 50 and 60°C and, (iii) optimal extension of the primed template at 70–78°C (Figure 10.1). A compact PCR format is recommended with some PCR assays which require only two steps: the denaturation and a combined annealing and extension step.

The oligonucleotide annealing temperature is usually referred to as the T_M (melting temperature) but is in fact 5–10°C below the T_M. The term T_M describes the temperature at which 50% of the oligonucleotide–target duplexes have formed. The T_M is dependent upon the concentration of the DNA, its length, nucleotide sequence and the composition of the solvent in which the DNA is suspended (Ririe *et al.*, 1997).
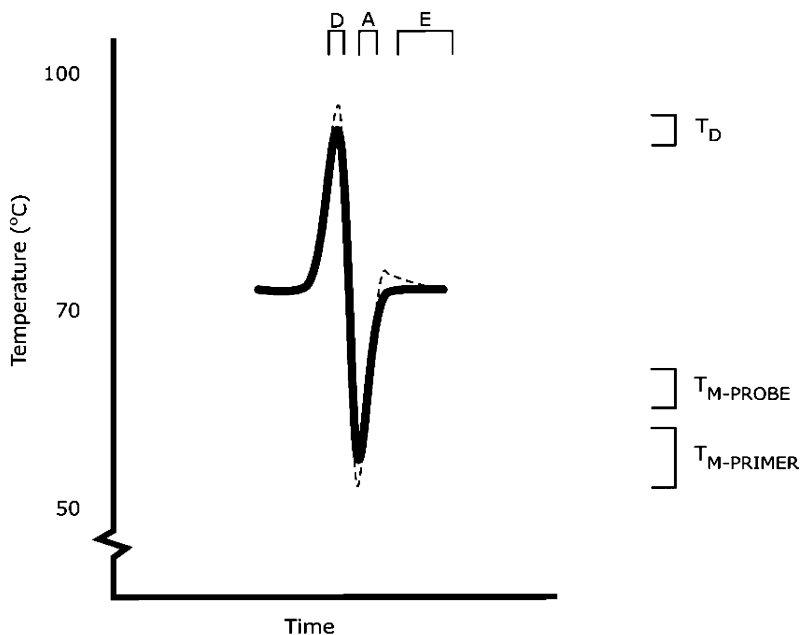
Figure 10.1. Time versus temperature plot during a single PCR Cycle. The denaturation (D), primer and probe annealing (A) and primer extension (E) steps are shown. At the indicated optimal temperature ranges, dsDNA denatures (T_D) then oligoprobes anneal ($T_{M\text{-PROBE}}$) followed by the primers ($T_{M\text{-PRIMER}}$) as a precursor to their extension. The actual thermal cycler incubation temperature (dashed line) may overshoot the desired temperature to varying degrees, depending on the quality of the thermal cycler employed.

The rate of temperature change in the reaction vessel, or ramp rate, the length of the incubation at each temperature and the number of times each cycle of temperatures is repeated, are all controlled by a programmable thermal cycler. Current technologies have significantly shortened the ramp rates and therefore total assay times, using electronically controlled heating blocks or fan-forced heated airflows.

Existing combinations of PCR and PCR product (amplicon) detection assays will be called "conventional PCR" throughout this chapter. These detection systems include analytical agarose gel electrophoresis (Kidd et al., 2000), Southern blot and ELISA-like systems such as ELAHA and ELOSA (van der Vliet et al., 1993; Chandelier et al., 2001; Mackay et al., 2001; Hyypiä et al., 1998). Traditional detection of amplified DNA relies upon its electrophoresis in the presence of ethidium bromide followed by visualisation or computer-assisted densitometric analysis of the bands during irradiation by ultraviolet light (Kidd et al., 2000). Whilst Southern blot detection of amplicon by hybridisation with a labelled oligonucleotide probe (oligoprobe) increases the specificity of amplicon detection, it is time consuming, frequently uses radioactive labels and requires multiple PCR product handling steps, increasing the risk of spreading amplicon throughout the laboratory (Holland et al., 1991). Alternatively, PCR–ELISA has been used to capture amplicon onto a solid phase via biotin or digoxigenin-labelled primers, oligoprobes or by direct capture after incorporation of the biotin or digoxigenin into the amplicon (van der Vliet et al., 1993; Keller et al., 1990; Kemp et al., 1990; Kox et al., 1996; Dekonenko et al., 1997; Watzinger et al., 2001). Once captured, amplicon is detected using an enzyme-labelled avidin or anti-digoxigenin reporter molecule in a manner similar to a standard ELISA format. PCR–ELISA has the added benefit of utilising hardware and techniques commonly available to the ELISA-enabled research and diagnostic microbiology laboratory.

The possibility that, in contrast to conventional PCR, real-time PCR could detect amplicon as it accumulated was welcomed by researchers and diagnostic scientists alike. This feat has expanded the view of PCR as a specialist tool to that of a versatile technology providing advanced and powerful systems for the research laboratory as well as permitting the development of contamination-resistant routine diagnostic applications for the clinical microbiology laboratory (Lomeli et al., 1989; Cockerill and Smith, 2002). Along the way, real-time PCR assays have provided insights into the nuts and bolts of the PCR as well as the performance of different nucleic acid extraction methods and the role some compounds play to inhibit amplification (Holland et al., 1991; Rosenstraus et al., 1998; Lee et al., 1993; Livak et al., 1995; Heid et al., 1996; Gibson et al., 1996; Niesters et al., 2000; Read, 2001; Biel et al., 2000; Petrik et al., 1997). Real-time PCR has allowed many more scientists to become familiar with the crucial factors contributing to successful amplification of nucleic acids and in its largest role to date, made the quantitation of gene transcription much simpler and faster. This has proven to be a valuable area of study since many cellular functions are regulated by changes in gene expression (Bustin, 2002; Balnaves et al., 1995). Today in microbiology, real-time PCR detects and quantifies nucleic acids from widely diverse targets including food,

viral and non-viral vectors used in gene therapy protocols, genetically modified organisms and to study human and veterinary microbiology, oncology and immunology (Böhm *et al.*, 1999; Kruse *et al.*, 1997; Stordeur *et al.*, 2002; Härtel *et al.*, 1999; Fraaije *et al.*, 2001; Nogva *et al.*, 2000; Barzon *et al.*, 2003; Klein, 2002; Rudi *et al.*, 2002; Mackay *et al.*, 2002; Ahmed, 2002; Mhlanga and Malmberg, 2001).

The monitoring of accumulating amplicon in real time has been made possible by the labelling of primers, oligoprobes or amplicon with molecules exhibiting fluorescent potential. The success of these assays revolves around a rapid and measurable signal change after the interaction of amplicon and fluorescent label (Morrison *et al.*, 1989). The signal is related to the amount of amplicon present during each cycle, increasing as the DNA is replicated. The fluorescent chemistries have clear benefits over earlier radiogenic labels including an absence of radioactive emissions, easy disposal and an extended shelf life (Matthews and Kricka, 1988).

Although some of the oligoprobe systems have been given a specific nomenclature by their developer, we will use the term "fluorophore" to describe the fluorescent moieties, and their inclusion on an oligonucleotide will imply that the resulting oligoprobe has fluorogenic properties.

◆◆◆◆◆◆ THE GOOD WITH THE BAD

Traditional diagnostic microbiological assays include microscopy, microbial culture and ELISA, which aim to detect microbial antigens or the host response to microbial presence. The performance of these assays can be limited by poor sensitivity, slowly growing or poorly cytopathic microbes, reduced microbial viability, narrow detection windows, complex result interpretation, host immunosuppression, antimicrobial therapies, high levels of background signal and non-specific cross-reactions (Whelan and Persing, 1996; Carman *et al.*, 2000). Nonetheless, microbial culture and rapid immunofluorescence assays are used to produce valuable epidemiological data, revealing new, uncharacterised or atypical microbes and yielding intact or infectious organisms for further study (Ogilvie, 2001). Although detection of microbial genomic nucleic acids is not identical to the detection of infectious particles, there is good correlation between infectivity and the viral genome as we have found for yellow fever virus (Bae *et al.*, 2003).

Fluorescence microscopy still remains a popular and rapid screening tool for samples containing large microbial loads whilst ELISA and related methods are ideal for identifying detectable levels of immune or cell function modulators (Lipson, 2002). It is, therefore, clear that the role of the traditional assay, be it in screening or research, continues to be an important one (Biel and Madeley, 2001; Pfaller, 1999; Sintchenko *et al.*, 1999; Ellis and Zambon, 2002; Clarke *et al.*, 2002; Johnson, 2000).

The PCR does have some significant limitations. Our ability to design oligonucleotide primers only extends to our knowledge of the sequence of the template (usually the genome of a microorganism or its host) as well

as the ability of publicly available sequence databases to suitably represent all variants of that target sequence. It is common for microbial genomes to contain unexpected "mutations", i.e. nucleotide changes compared to known microbial sequences, which reduce or abrogate the function of a PCR. Additionally, it is often the effects of these variations on colonisation and pathogenesis which warrant further study. Each PCR assay requires careful optimisation of several variables, ensuring that primer and magnesium concentrations are perfected as well as selecting the best polymerase for the assay (Kreuzer *et al.*, 2000; Wolffs *et al.*, 2004). Also, new batches of reagents must be carefully tested against previous batches (Burgos *et al.*, 2002). This testing is especially important for oligonucleotides since all manufacturers have "bad days" that equate to wasted time and added frustration for the assay developer. The use of standardised assay panels and the introduction of international units have begun to improve microbial diagnostics for the limited number of pathogens these encompass (Valentine-Thon, 2002).

False positive results due to amplicon carry-over contamination have always been a major concern for the routine implementation of PCR in the diagnostic laboratory and this has led to strict guidelines for laboratory design and work flow (McCreedy and Callaway, 1993). There are also occasions when the PCR is too sensitive, detecting a microbe that is present below pathogenic levels. Thus, care is required not only for assay design but also for result interpretation.

Real-time PCR assays also carry specific disadvantages compared to conventional PCR, including the inability of certain platforms to detect some fluorogenic chemistries and the relatively restricted multiplex capabilities of current systems. Also, the start-up expense of real-time PCR instruments is prohibitive for many low-throughput laboratories and ongoing costs are higher when compared to agarose gel amplicon detection although lower than many PCR–ELISA formats.

A significant improvement introduced by real-time PCR is the increased speed with which results can be produced. This is largely due to the removal of a separate post-PCR detection step, the use of sensitive fluorescence-detection equipment which permits earlier amplicon detection and the shortened cycle times possible on some instruments (Wittwer *et al.*, 1990, 1997b). A reduced amplicon size may also increase assay speed, however, we and others have shown that decreased product size does not strictly correlate with improved PCR efficiency but the distance between the primers and the oligoprobe does play a vital role (Nitsche *et al.*, 2000; Balnaves *et al.*, 1995; Lunge *et al.*, 2002).

◆◆◆◆◆◆ GENERATING A FLUORESCENT SIGNAL

Most of the popular real-time PCR chemistries involve hybridisation of one or more fluorescent oligoprobes to a complementary sequence on one of the amplicon strands. Therefore, the inclusion of more of the primer that creates the strand complementary to the oligoprobe – a process called

asymmetric PCR – is often beneficial to the generation of an increased fluorescent signal (Gyllensten and Erlich, 1988; Barratt and Mackay, 2002). However, it is critical to determine which strand to target, particularly in the case of single stranded genomes such as those harboured by negative or positive sense viruses.

As mentioned earlier, post-amplification manipulation of the PCR product is not necessary to detect amplicon using real-time PCR because the fluorescent signals are directly measured as they exit the reaction vessel. Therefore, real-time PCR assays are often described as homogeneous or "closed" systems; combining template amplification and amplicon detection into a single reaction. Apart from the time saved by amplifying and detecting template in a single tube, there is minimal potential for amplicon carry-over contamination and the assay's performance can be closely scrutinised without introducing errors due to handling of the PCR product (Higuchi *et al.*, 1993). An often overlooked benefit to the use of real-time PCR is its overall cost effectiveness on a per-run basis, when implemented in a high-throughput laboratory (Martell *et al.*, 1999), particularly when replacing conventional PCR and gel or probe-based amplicon detection systems or microbial culture.

The most commonly used fluorogenic oligoprobes rely upon fluorescence resonance energy transfer (FRET; Figure 10.2) 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 (Didenko, 2001). FRET is a non-radiative process in which energy is passed between permissive molecules that are spatially separated by 10–100 Å and which have overlapping emission and absorption spectra (Stryer and Haugland, 1967; Heller and Morrison, 1985; Clegg, 1992). Förster primarily developed the theory behind this process (Förster, 1948). The energy transfer reduces the lifetime of the excited state of electrons in the original fluorophore by taking the emitted excess energy and expelling it either as heat or fluorescence. 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 (Selvin, 1995; Didenko, 2001). FRET permits the determination of nucleic acid hybridisation, without the prior removal of unbound probe required for other hybridisation techniques (Cardullo *et al.*, 1988).

Fluorescence data generated by real-time PCR assays are generally collected from PCR cycles that occur early in the reaction where amplification conditions are optimal and the fluorescence accumulates in proportion to the amplicon (Figure 10.3). Because the emissions from fluorescent chemistries are temperature-dependent, data is generally acquired only once per cycle, at the same temperature (Wittwer *et al.*, 1997a). Signal detection occurring at the end point of the reaction is not ideal since amplicon accumulation may have been adversely affected by inhibitors, poorly optimised reaction conditions or saturation effects caused by excess double-stranded amplicon. In fact at the end point there may be no relationship between the initial template and final amplicon concentrations (Figure 10.3).
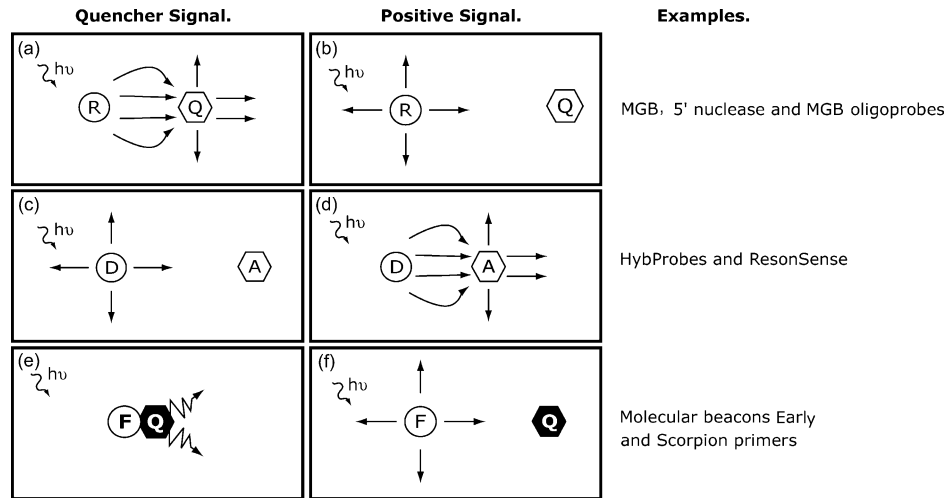
Figure 10.2. Mechanisms of fluorescence resonance energy transfer (FRET). When the reporter (R) and quencher (Q, unfilled) of a nuclease oligoprobe are in close proximity and illuminated by an instrument's light source (where h is Planck's constant and ν is the frequency of the electromagnetic radiation); (a) the quencher "hijacks" the emissions from excitation of the reporter. The quencher then emits this energy. When the fluorophores are separated, as occurs upon oligoprobe hydrolysis as depicted in (b), the quencher can no longer influence the reporter which now fluoresces at a distinctive wavelength recorded by the instrument. In the reverse process using adjacent oligoprobes (c), the fluorophores begin the cycle as separated entities. Whilst the emission of the donor (D) is monitored, it is the signal from the acceptor (A) produced when in close proximity to the donor that indicates a positive reaction (d). In (e), another form of quenching is shown, caused by the intimate contact of labels attached to hairpin oligoprobes. The fluorophore (F) and an NFQ (Q, filled) 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, arrows). When the labels are separated, as is the case in (f), the fluorophore is free to fluoresce.
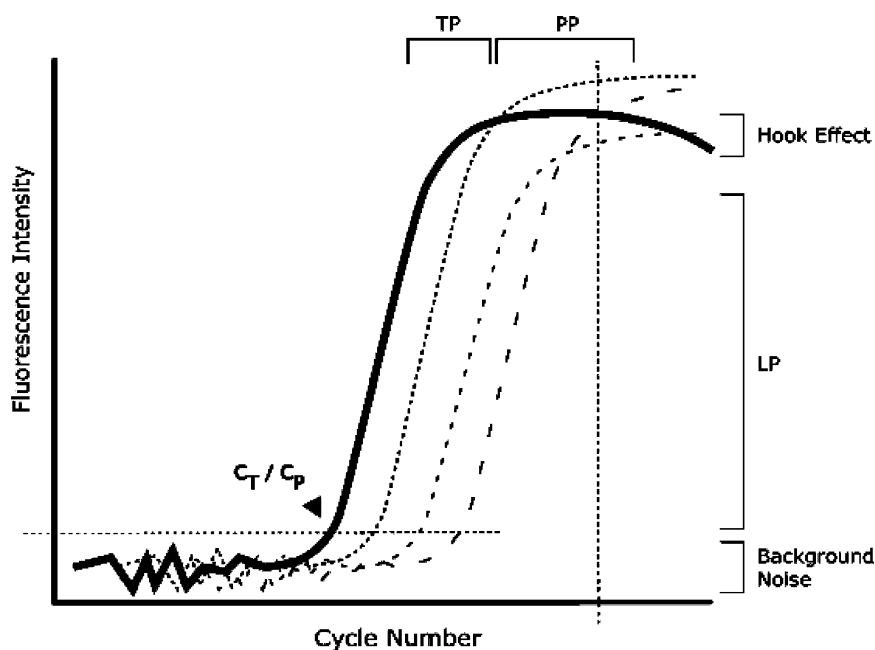
Figure 10.3. Kinetic analysis of amplicon accumulation. A chart of the amplification of a template by real-time PCR (solid line) ideally appears as a sigmoidal curve when plotted as cycle number versus fluorescence emission intensity. Early exponential amplification cannot be viewed because the signal is below the sensitivity of the detector. 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 one $\log_{10}$ every 3.32 cycles (i.e. it doubles, or increases by one $\log_2$ every cycle). As primers and enzymes become limiting and products accumulate that are inhibitory to the PCR or compete for hybridisation with an oligoprobe, the reaction slows, entering a transition phase (TP). Eventually a plateau phase (PP) is reached in which there is little or no increase in fluorescent signal although amplicon may continue to accumulate. Some fluorescent detection chemistries display an overall reduction in fluorescence intensity after the plateau phase (Hook). The point at which fluorescence surpasses a pre-defined background noise (dashed horizontal line) is called the threshold cycle or crossing point (C_T or C_P; indicated by an arrow). These data are used for the calculation of template quantity when constructing a standard curve. Traditional PCR data collection is performed at the end of the assay (dashed vertical line). Also shown are curves representing a serial titration of template (dashed curves), consisting of decreasing starting template concentrations, which produce increasing numerical C_T or C_P values.

The fractional cycle number at which the real-time fluorescence signal mirrors progression of the amplification reaction above the background noise level is used as an indicator of successful target amplification (Wilhelm *et al.*, 2001a). Most commonly, this is called the threshold cycle (C_T) but the same value is described for use with the LightCycler where the fractional cycle is called the crossing point (C_P). The C_T is defined as the PCR cycle at 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

(Jung *et al.*, 2000). The C_T and C_P are proportional to the number of template copies present in the sample (Gibson *et al.*, 1996). The values are assumed to represent equal amounts of amplicon in each vessel at the time of measurement, since the C_T and C_P values are acquired from a single fluorescence intensity value (Figure 10.3). In practice C_T and C_P are calculated after the definition of a noise band which excludes data from early PCR cycles that cannot be distinguished from background fluorescence. Therefore the final C_T and C_P value is the fractional cycle at which a specimen's plotted PCR curve intersects a single fluorescence value (usually at or close to the noise band; Figure 10.3, Wilhelm *et al.*, 2001a). The accuracy of the C_T or C_P depends upon the concentration and nature of the fluorescence-generating system, the amount of template initially present, the sensitivity of the fluorescence detector and its ability to discriminate specific fluorescence from background noise.

◆◆◆◆◆◆ REAL-TIME INSTRUMENTATION

Broadly speaking, real-time PCR instruments (Table 10.1) can be divided into two classes based on the method used to heat the reaction vessel: the block thermal cyclers and the air thermal cyclers. Block cyclers generally ramp at 1–10°C/s whereas heated air cyclers can ramp as quickly at 20°C/s. The faster rates permit 10–20 min assays (excluding hands-on time); however, these times are infrequently reported for microbial real-time PCR assays, probably due to technical difficulties when applied to clinical specimens under real-life conditions. An off-shoot of the block cyclers are miniaturised thermal cyclers capable of performing real-time PCR but constructed of sturdy solid-state and relatively low-cost materials permitting transport of the PCR laboratory onto the site of testing (Northrup, 1998; Ibrahim *et al.*, 1998).

The quality of the instrument has a significant role in the reproducibility of results, which subsequently influences how well a described protocol transports between laboratories around the world. A common discrepancy between systems is the temperature profile of a PCR cycle (Schoder *et al.*, 2003). Since temperature affects enzyme function, fluorescence and oligonucleotide binding, it is an important consideration during the purchase of a real-time PCR system.

The majority of PCR thermal cyclers use capped plastic reaction vessels to house the amplification reaction. However, the LightCycler (Roche Molecular Biochemicals, Germany) differs by using glass capillaries (Wittwer *et al.*, 1997a). The LightCycler's plastic and glass composite capillaries are optically clear and act as cuvettes for fluorescence analysis, as well as facilitating rapid heat transfer. However, they are fragile and require some experience to handle (Schalasta *et al.*, 2000a). For the Roche LightCycler and the Corbett Robotics RotorGene, the temperature is varied by rapidly heating and cooling air using a heating element and fan which produce ramp rates of 2.5 and 20°C/s, respectively.

Table 10.1. Popular real-time PCR instrumentation

Brand	Company	Light source	Capacity	Heating method	Real time
LightCycler	Roche	LED	32	Air	✔
iCycler	BioRad	Halogen	96–384	Block	✔
Mx4000	Stratagene	Halogen		Block	✔
RotorGene	Corbett	LEDs	36–72	Air	✔
7700 (discontinued)	Applied Biosystems	Laser	96	Block	✔
7900HT	Applied Biosystems	Laser	96	Block	✔
7000	Applied Biosystems		96	Block	✔
Chimaera	Hybaid	Halogen	96	Block	✔
DNA Engine Opticon 2	MJ Research	LEDs	96	Block	✔
Smartcycler	Cepheid	LEDs	16	Block	✔

Real-time Fluorescent PCR
Techniques to Study

Faster rates prolong polymerase survival and significantly shorten the assay's completion time but existing assays may require some fine tuning (Weis *et al.*, 1992).

In the following sections, we will focus on real-time PCR performance especially the rapidly expanding range of real-time PCR chemistries. Also, we will review the application of real-time PCR to determine the interplay between microbe and host via microbial diagnosis and quantitation. We will also describe the limitations imparted upon real-time PCR by instrument design and the paucity of fluorophores. Although this review focuses on the application of fluorogenic chemistries to real-time PCR applications, they are also valuable as fluorogenic end-point amplicon detection assays. In these instances the ability to collect data as the amplicon accumulates, i.e. kinetic analysis, is lost but the systems retain a homogeneous format.

◆◆◆◆◆◆ DETECTING AMPLICON USING FLUORESCENCE

It is the amplicon detection processes that discriminate real-time PCR from conventional PCR assays. There are a range of chemistries currently in use and these can be broadly categorised as amplicon specific or non-specific (Whitcombe *et al.*, 1999). To further clarify, the amplicon itself is produced from a PCR using sequence-specific primers. However, the specificity we refer to is that of amplicon detection. The difference arises from the use of a fluorophore that interacts with any and all dsDNA such as SYBR® green I, or the use of a sequence-specific, fluorogenic oligoprobe. In general, however, the specific and non-specific fluorogenic chemistries detect amplicon with equal sensitivity (Wittwer *et al.*, 1997b) and have recently been reviewed in detail (Mackay *et al.*, 2002). New fluorogenic systems continue to be described but few applications for the specific detection, quantitation and genotyping of microbes using these recent chemistries have been reported. Undoubtedly some of these new chemistries are the result of pure research projects; however, it is tempting to propose that at least some of these chemistries have been developed in order to circumvent patents rather than to significantly advance the technology.

DNA-associating Fluorophores

The DNA-associating fluorophore is the basis of the non-specific real-time PCR detection methods. Many of these molecules interact with dsDNA by associating with the minor groove of the DNA duplex. As a group, these fluorophores display minimal fluorescence when free in solution, but fluoresce strongly when associated with nucleic acids which occur with high affinity. The simplest real-time PCR reporter systems are included within the DNA-associating fluorophores. Ethidium bromide

(Higuchi *et al.*, 1992), YO-PRO-1 (Ishiguro *et al.*, 1995; Tseng *et al.*, 1997), SYBR® green I (Morrison *et al.*, 1998b), SYBR Gold (Tuma *et al.*, 1999), BEBO (Bengtsson *et al.*, 2003) and LCGreen (Vaisse *et al.*, 1996) are all molecules which fluoresce when associated with dsDNA which is exposed to a wavelength of light capable of exciting the fluorophore. This approach to detection is inexpensive and simpler than the design requirements of fluorogenic oligoprobes and is minimally affected by small changes in template sequence which may abrogate hybridisation of an oligoprobe (Komurian-Pradel *et al.*, 2001). However, recent evidence suggests that SYBR green I preferentially binds to amplicon species that melt at higher temperatures, indicating a preference for $G + C$-rich regions, which would prove detrimental to its use in multiplex assays (Giglio *et al.*, 2003). Possibly, SYBR Green I binds to high-temperature duplexes after melting off lower temperature duplexes, resulting in a hierarchy of melting peak heights expressing no relation to starting template concentration (Vaisse *et al.*, 1996). A recent addition to this chemistry, LCGreen, displays a higher sensitivity for lower temperature duplexes (Vaisse *et al.*, 1996).

Primer–dimer formation is common and is strongly associated with the entry of the PCR into a plateau phase of amplification (Figure 10.3; Chou *et al.*, 1992; Halford, 1999; Halford *et al.*, 1999). Association of a DNA-binding fluorophore with primer–dimer or with another non-specific amplification product can confuse interpretation of the PCR results. The problem of primer–dimer can be addressed using software capable of fluorescent melting curve analysis (FMCA). We will describe the use of FMCA for genotyping in a later section (Figure 10.18); however, in the context of SYBR green I, FMCA is completed in minutes, requires no amplicon manipulation and utilises the temperature at which a dsDNA amplicon denatures or "melts" (T_D; Figure 10.1). The shorter primer–dimer can theoretically be discriminated by its reduced T_D compared to the full-length amplicon. Practically, this discrimination is heavily reliant upon the $G + C$ content of both the specific amplicon and any primer–dimer, as well as the length of the amplicon. Analysis of the melting character of an amplicon in the presence of SYBR green I has shown that the sensitivity of DNA-binding fluorophores is limited by non-specific amplification at low initial template concentrations (Wittwer *et al.*, 1997a). Unfortunately, limited template is a frequent occurrence when analysing patient specimens. The contribution of non-specific products to the accumulating fluorescence signal can be reduced in many instances by selecting a data collection temperature above the known T_D of the unwanted products (Morrison *et al.*, 1998a). However, there is no reliable real-time PCR solution to discriminate non-specific amplicon when it has a similar or higher T_D than the target amplicon species.

DNA binding fluorophores increase the T_D and broaden the melting transition, requiring substantial sequence change to produce a noticeable shift in the T_D compared to using the T_M to discriminate nucleotide polymorphisms. Oligoprobes permit the clear discrimination of single point mutation using the T_M (Wetmur, 1991). This is especially useful for discriminating related microbes.

Fluorogenic Oligoprobe and Primer Chemistries

The adoption of oligoprobes has added an additional layer of specificity to the PCR by confirming the sequence of the amplicon, in addition to the binding of a specific pair of primers. By using an excess of oligoprobe, the time required for it to hybridise with its target is significantly reduced (Wetmur, 1991). This is especially so when the amount of that target has been increased by PCR or some other molecular amplification process (Morrison *et al.*, 1989).

While the most common oligoprobes are based on conventional nucleic acid chemistry, peptide nucleic acids (PNA) are becoming an increasingly popular choice for oligonucleotide backbones. PNA are a DNA analogue formed from neutral, repeated *N*-(2-aminoethyl) glycine units instead of the negatively charged sugar phosphates of DNA (Egholm *et al.*, 1993). The PNA oligoprobe retains the same sequence recognition properties of DNA but it cannot be extended or hydrolysed by a DNA polymerase. A more recent family of DNA analogues are the locked nucleic acids (LNA; $2'$-*O*,$4'$-*C*-methylene-β-D-ribofuranosyl) (Singh *et al.*, 1998; Koshkin *et al.*, 1998; Obika *et al.*, 1998; Kumar *et al.*, 1998; Petersen and Wengel, 2003). LNA are modified nucleic acids in which the sugar has been conformationally "locked", imparting unprecedented hybridisation affinity towards DNA and RNA. LNA monomers can be added to a synthetic oligonucleotide as desired using conventional phosphoramidite chemistry and their addition increases the thermal stability of the oligo permitting the construction of shorter oligoprobes for real-time PCR applications (Simeonov and Nikiforov, 2002).

To be optimal, an oligonucleotide label must easily attach to DNA and be detectable at low concentrations. The label or labels should produce an altered signal upon specific hybridisation, remain biologically innocuous and be stable at elevated temperatures. Additionally, the label should not interfere with the activity of the polymerase (Matthews and Kricka, 1988; Holland *et al.*, 1991).

General considerations for the design of a fluorogenic oligoprobe should include a length of 20–40 nt with a G + C content of 40–60%. The oligoprobe should neither contain clusters of a single nucleotide, particularly G, nor should it have repeated sequence patterns nor hybridise with the forward or reverse primers. A fluorescent label should efficiently absorb and emit energy and release its emissions at dissimilar wavelengths so that excitation and emission can occur concurrently. The relationship between a fluorescence signal indicating positive hybridisation and a signal from unwanted fluorescence is often referred to as the signal-to-noise ratio (signal:noise). A high signal:noise is preferred. Generally speaking, this can best be achieved by oligoprobe chemistries that utilise an NFQ and function through a unimolecular signalling configuration, i.e. only one molecule is required to directly indicate the presence of amplicon.

Deoxyguanosine nucleotides (G) naturally quench some fluorophores (e.g. FITC) in a position-dependent manner (Crockett and Wittwer, 2001). Natural quenching varies linearly with a defined concentration range

of template. The level of quenching can be increased if more guanines are present or if a single guanine is located in the first non-hybridised position of the oligoprobe:amplicon duplex. In this position, the G will be located one nucleotide beyond the fluorophore-labelled terminus of the probe, where it will remain free once the oligoprobe has hybridised to the target amplicon. A hybridised G does not quench to the same degree as a free G. This knowledge has been applied to some amplicon detection approaches since a single labelled oligonucleotide is easy to design and use and relatively simple to synthesise. In addition, this approach does not require a DNA polymerase with nuclease activity (Crockett and Wittwer, 2001).

Ideally, an oligoprobe should have a T_M at least 5°C higher than that of the primers, to ensure the oligoprobe(s) hybridises with its template before extension of the primers begins (Landt, 2001). This caveat also applies to each component of a multi-oligonucleotide fluorogenic chemistry requiring two or more hybridisation events for effective signalling such as the tripartite molecular beacons (TMB), universal template (UT) oligoprobe or duplex scorpion primers.

All the non-incorporating, nucleotide-based, oligoprobe chemistries described in the following sections include a 3′ phosphate or similar moiety, which blocks their extension by the DNA polymerase preventing the oligoprobe acting as a primer, but imparting no effect on the amplicon's yield. These fluorogenic chemistries can be divided into two classes: those which are destroyed to produce fluorescence and those which are not. Oligoprobes depending on a destructive process for signal generation are usually located close to the primer that hybridises to the same strand in order to maximise the chance that polymerase will make contact with the bound oligoprobe. Non-destructive oligoprobes are usually located as far as possible from the primer on the same strand to ensure signal is produced before the polymerase dislodges the duplex. The non-destructive chemistries include linear and hairpin oligoprobes, and incorporating primers. While reviewing these options one should note the number of hybridisation events required to generate a positive signal. Each fluorogenic oligonucleotide must meet and hybridise with its specific amplicon (a bimolecular system). If there is a second oligoprobe required, either to provide a quenching moiety or as a partner for FRET, the likelihood of a chance encounter between all three molecules decreases (a trimolecular system), especially as the amplicon concentration increases. Conversely, the fluorogenic primer systems are incorporated into the nascent amplicon. Therefore, a signal is generated for the remainder of the assay without the need for hybridisation to a new amplicon molecule each cycle (a unimolecluar system).

Destructive oligonucleotide systems

In 1991, Holland *et al.* described a technique that was to form the foundation for homogeneous PCR using fluorogenic oligoprobes. Radiolabelled amplicon was detected by monitoring the 5′ to 3′ nuclease activity of *Taq* DNA polymerase on specific oligoprobe and target DNA

duplexes. The products were examined using thin layer chromatography and the presence or absence of hydrolysis was used as an indicator of specific duplex formation.

Lee et al. (1993) reported an innovative approach using nick-translation PCR in combination with a dual-fluorophore labelled oligoprobe. In the first truly homogeneous assay of its kind, one fluorophore was added to the 5′ terminus and one to the middle of a sequence-specific oligoprobe. When in such close proximity, the 5′ reporter fluorophore (6-carboxy-fluorescein, FAM) transferred laser-induced excitation energy by FRET to the 3′ quencher fluorophore (6-carboxy-tetramethyl-rhodamine, TAMRA). TAMRA emitted the new energy at a wavelength that was monitored but not specifically utilised in the presentation of data. However, when the oligoprobe is hybridised to its template, the fluorophores were released upon hydrolysis of the oligoprobe component of the probe and target duplex due to the nuclease activity of the DNA polymerase. Once the labels were separated, the reporter's emissions were no longer quenched and the instrument could detect and present the resulting fluorescence data. These oligoprobes have been called 5′ nuclease, hydrolysis or TaqMan® oligoprobes (Figure 10.4).

The use of 5′ nuclease probes first required the development of a platform that could excite and detect fluorescence in addition to thermal cycling. A charge-coupled device (CCD) was described in 1992 for the quantification of conventional reverse transcription (RT)-PCR products (Nakayama et al., 1992). In 1993, the CCD was combined with a thermal cycler resulting in the first real-time PCR fluorescence excitation and detection instrument (Higuchi et al., 1993). To date, the commercial version of the platform, the ABI Prism® 7700 sequence detection system (Perkin Elmer Corporation/Applied Biosystems, USA), has been the most frequently reported instrument used with 5′ nuclease oligoprobes.

A recent addition to the destructive chemistry is the UT oligoprobe (Figure 10.5; Zhang et al., 2003). This system adds a generic or "universal" sequence to the 5′ end of a PCR primer with which a common 5′ nuclease oligoprobe can hybridise permitting the use of the same oligoprobe for different amplicons. The remainder of the primer provides assay specificity so that multiple primers can be used to amplify numerous targets all detected using the same oligoprobe. During the second PCR cycle, the nascent strand is copied and the polymerase encounters and hydrolyses the bound oligoprobe releasing the reporter and permitting fluorescence to be generated. Because the oligoprobe relies upon hybridisation with a primer, it is possible that this chemistry will also produce fluorescence from the formation of non-specific amplicon.

Another dual-labelled oligonucleotide sequence has been used as the signal-generating portion of the DzyNA-PCR system (Figure 10.6; Todd et al., 2000; Applegate et al., 2002). The reporter and quencher are attached to the termini of an oligonucleotide substrate. Cleavage of the oligosubstrate is performed by a DNAzyme, which is created during the PCR. This creation is the result of the PCR duplicating an antisense DNAzyme sequence included in the 5′ tail of one of the primers. The duplicated sequence is the "functional" form of the DNAzyme.
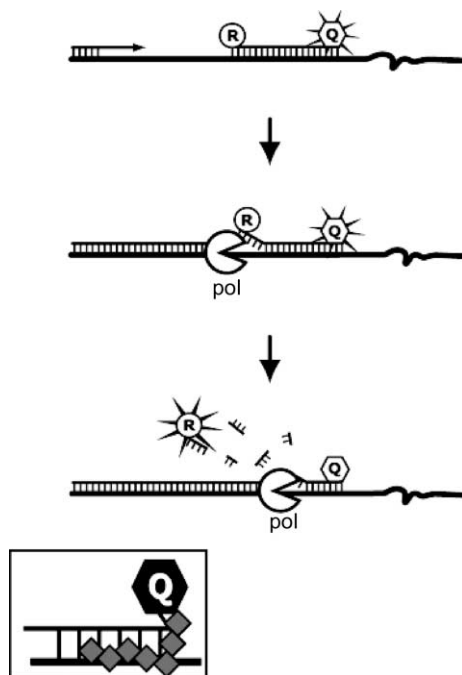
Figure 10.4. Function of 5′ nuclease oligoprobes. Following primer hybridisation, the DNA polymerase (pol) progresses along the relevant strand during the extension step of the PCR, displacing and then hydrolysing the oligoprobe. Once the reporter (R) is removed from the extinguishing influence of the quencher (Q), 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 (Q; filled) and minor groove-binding molecule (grey diamonds) which make up the MGB nuclease oligoprobes. These bimolecular systems acquire data from the reporter's emissions: the opposite of the HybProbe chemistry. Data can be collected during the annealing or extension steps of the PCR.

Upon cleavage, the fluorophores are released allowing the production of fluorescence in an identical manner to a hydrolysed 5′ nuclease oligoprobe.

Non-destructive oligonucleotide systems

Linear chemistries

The majority of fluorogenic oligoprobes fall into the class of linear oligoprobes. In fact, the use of a pair of adjacent, fluorogenic oligoprobes was first described in the mid-1980s, predating the popular 5′ nuclease chemistry but failing to achieve the same degree of early commercial support. The pair of oligonucleotides were used to identify the distance between fluorophores on a complementary nucleic acid template and the system held promise for similar detection within living cells, hinting at further possibilities for diagnostic use (Heller and Morrison, 1985; Cardullo *et al.*, 1988). This trimolecular approach is now known
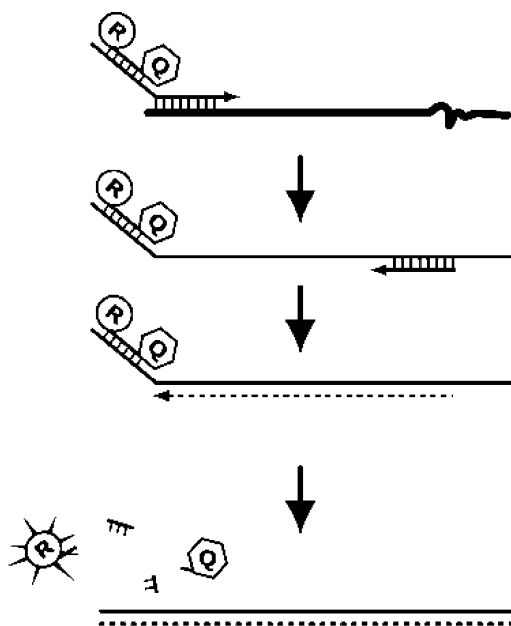
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Figure 10.5. Function of the UT-oligoprobe. After the UT-primer is extended, the nascent strand acts as the template for the second primer. The polymerase encounters and hydrolyses the UT-oligoprobe whilst extending the second primer in the same fashion as a TaqMan oligoprobe. This trimolecular system can produce fluorescence data from the emissions of the released fluorophore during the annealing or extension steps.

commercially as hybridisation probes (HybProbes), having become the manufacturer's chemistry of choice for the LightCycler™ (Roche Molecular Biochemicals, Germany; Wittwer *et al.*, 1997b). The upstream oligoprobe is labelled with a $3'$ donor fluorophore (FITC) and the downstream probe is commonly labelled with either a proprietary LightCycler Red 640 or a Red 705 acceptor fluorophore at the $5'$ terminus with a phosphate at the $3'$ terminus to prevent extension by the DNA polymerase. When both oligoprobes are hybridised to the amplicon template, the two fluorophores are ideally located within 10 nt of each other (Figure 10.7). The ratio of acceptor to donor emissions can also be used as an internal reference signal rendering the results independent of absolute fluorescence (Huang *et al.*, 2001).

The double-stranded oligoprobes function by displacement hybridisation (Figure 10.8; Li *et al.*, 2002). 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-labelled with an NFQ. When the full-length complementary sequence in the form of an amplicon is generated, the reporter strand preferentially hybridises to the longer target amplicon strand, disrupting the quenched oligoprobe duplex and permitting the fluorophore to emit its excitation energy directly.

The displacement hybridisation technique can also be used with a fluorophore-labelled primer and, due to added stringency provided by the longer complementary strand, the system performs its own
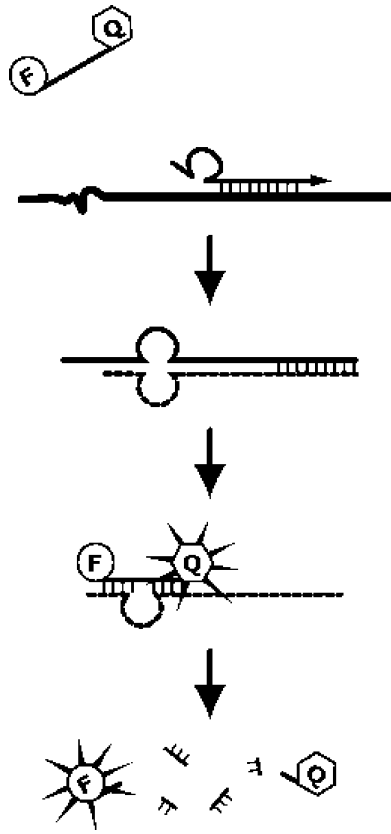
Figure 10.6. Function of the DzyNA primers. When the strand incorporating the primer is duplicated by a complementary strand (dashed line), a DNAzyme is created. A complementary, dual-labelled oligonucleotide substrate will be specifically cleaved by the DNAzyme releasing the fluorophore (F; circle) from its proximity to the quencher (Q; pentagon), releasing the labels and permitting fluorescence. Data can be collected during the annealing or extension steps of the PCR.
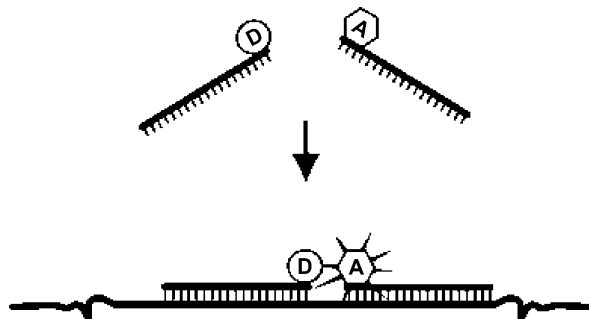
Figure 10.7. Function of HybProbes. Adjacent hybridisation results in a FRET signal due to interaction between the donor (D) and acceptor (A) spectra detected during the annealing step of the PCR. This trimolecular system (two oligoprobes and a target) acquires its data from the acceptor's emissions: the opposite of the 5′ nuclease oligoprobe chemistry.
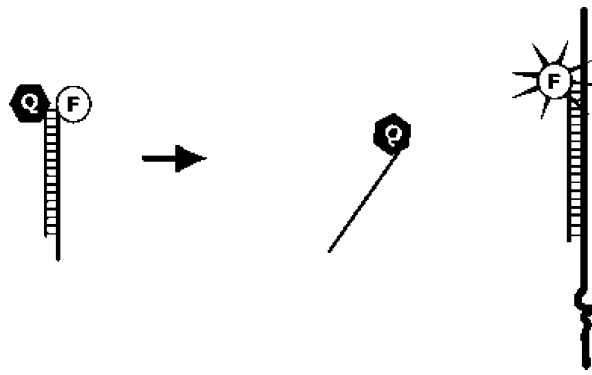
Figure 10.8. Function of displacement oligoprobes. The shorter NFQ-labelled strand (Q; hexagon) is displaced when the fluorophore-labelled (F; circle) strand preferentially hybridises to the longer specific amplicon strand. Data is collected from this trimolecular system during the annealing step of the PCR.

"hot-start" as was shown using an NFQ-labelled PNA strand (Q-PNA; Figure 10.9; Stender *et al.*, 2002; Fiandaca *et al.*, 2001). This and the previous chemistry are effectively trimolecular systems since the quenching strand must re-anneal after dissociating with amplicon in order to complete the signalling process. In the Q-PNA approach, a short quenching PNA probe is bound to an unincorporated fluorogenic primer such that the NFQ and fluorophore are adjacent, resulting in a quenched system. However, once the dsDNA amplicon is created by primer extension, the shorter Q-PNA is displaced in favour of the longer target amplicon, after which the fluorophore can fluoresce.

The first generation Light-up probe (Light-Up Technologies) is also a linear PNA, and is labelled with an asymmetric cyanine fluorophore, thiazole orange (Figure 10.10; Svanvik *et al.*, 2001). When hybridised with
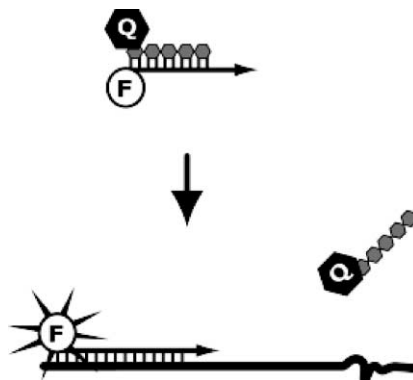

Figure 10.9. Function of the Q-PNA displacement primer. In the absence of the longer specific amplicon, quenching of the chemistry is achieved by a short NFQ-labelled PNA backbone (grey hexagons) designed to hybridise with the fluorophore-labelled primer (F; circle). Fluorescence data can be collected from this trimolecular system during the annealing and extension steps of the PCR.
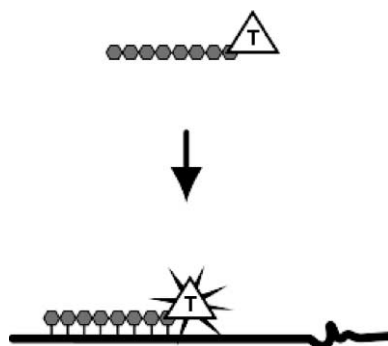
Figure 10.10. Function of the Light-Up Probe. These PNA (grey hexagons) oligoprobes fluoresce when their asymmetric thiazole orange fluorophore (T; open triangle) hybridises to the specific DNA strand. Data is collected from this bimolecular system during the annealing step of the PCR.

a nucleic acid target, either as a duplex or triplex, the fluorophore emits fluorescence due to association with amplicon. These probes do not interfere with the PCR, do not require conformational change and they are sensitive to single nucleotide mismatches, permitting further amplicon characterisation. The system is bimolecular 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 (Svanvik *et al.*, 2001; Isacsson *et al.*, 2000). However, non-specific fluorescence has been reported during extended cycling (Svanvik *et al.*, 2000).

The HyBeacon™ is a single linear oligonucleotide internally labelled with a fluorophore that emits an increased signal upon formation of a duplex between the target DNA strand and the HyBeacon (Figure 10.11; French *et al.*, 2001, 2002). The HyBeacon and Light-up probe are relatively inexpensive and simple bimolecular systems to design and use.
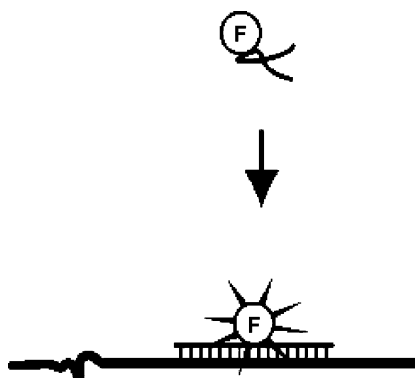

Figure 10.11. Function of the HyBeacon Oligoprobe. The fluorophore (F; circle) emits fluorescence when in close proximity to DNA as occurs upon hybridisation with the specific amplicon strand. Data is collected from this bimolecular system during the annealing step of the PCR.

The dual-labelled PNA was produced briefly as the Lightspeed probe or linear PNA beacon by Boston Probes Inc., USA (Stender *et al.*, 2002). The oligoprobe is analogous to the TaqMan chemistry terminally labelled with a fluorophore and quencher, but it differs in its backbone, which is PNA (Figure 10.12). In an aqueous solution the PNA backbone brings the fluorophore and quencher into close proximity, quenching the system. When the probe hybridises to its specific target, it opens and fluorescence is possible.

A modification to the $5'$ nuclease chemistry resulted in the minor groove binding (MGB) oligoprobes (Figure 10.4, inset; Afonina *et al.*, 2002a). This chemistry has a fluorescent reporter dye at the $5'$ end and a NFQ at the $3'$ end. In addition, the oligoprobe has an MGB molecule at the $3'$ end that hyper-stabilises each oligoprobe–target duplex by folding into the minor groove of the dsDNA (Kutyavin *et al.*, 2000; Afonina *et al.*, 2002b). In the unbound state the oligoprobe assumes a random coil configuration that is quenched. When hybridised, the stretched oligoprobe is able to fluoresce. However, as with many dual-labelled oligoprobe systems, this relaxed state can "leak" fluorescence at higher temperatures when the oligoprobe is prone to partial unfolding. When using $5'$ nuclease oligoprobes we found that this leakage can even produce a melting peak under controlled conditions (Mackay *et al.*, 2003b).

The MGB chemistry permits the use of very short (12–17 nt) oligoprobes because of a 15–30°C rise in their T_M resulting from the interaction of the MGB and the DNA helix, in particular its stabilisation of A:T bonds. These short oligoprobes are theoretically ideal for detecting single nucleotide polymorphisms, since a short oligoprobe is more significantly destabilised by changes within the hybridisation site than a longer oligoprobe. However, in practice the degree of discrimination depends on the base to be determined, since some nucleotides can significantly influence fluorescence intensity. Originally this approach was designed
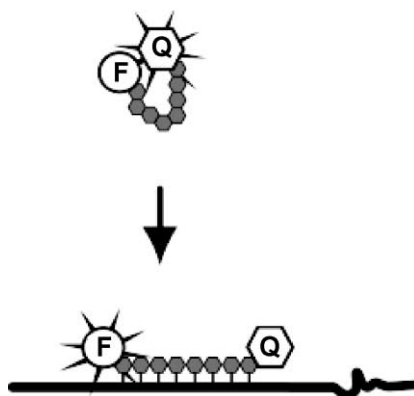

Figure 10.12. Function of the Lightspeed probe. In aqueous solution the PNA (grey hexagons) probe forms a random coil conformation that is quenched due to the proximity of the fluorophore (F; circle) and quencher (Q; hexagon). Upon hybridisation this bimolecular system is stretched open and the fluorophore can emit fluorescence which is acquired during the annealing step of the PCR.

for the allelic discrimination of genomic DNA where the ratio of heterozygotes to wild-type sequence was 1:1. In situations where this ratio is significantly skewed, the discriminatory power of polymorphism detection by oligoprobe may be reduced. A similar chemistry is the commercial MGB-Eclipse™ oligoprobe, which replaces the standard TAMRA or DABCYL quencher with a proprietary NFQ at the 5′ and a fluorophore at the 3′ end. The MGB molecule protects the oligoprobe from degradation by the polymerase and instead the signal is generated by stretching and relaxing of the oligoprobe during annealing and melting.

The result of combining a single sequence specific, Cy5-labelled linear oligoprobe with SYBR green I created the Bi-probe system. This functions via a variation of FRET recently termed Induced FRET (iFRET; Cardullo *et al.*, 1988; Howell *et al.*, 2002). Bi-probes are more specific than using SYBR green I alone and have an enhanced signal:noise (Brechtbuehl *et al.*, 2001; Walker *et al.*, 2001). Interestingly this approach functions using the Idaho Technologies LightCycler but not the closely related Roche LightCycler version 1.0. The disparity is due to the narrow bandpass filter sets employed by the latter instrument, which prohibited FRET between these two particular fluorophores. A similar technical problem rendered some proprietary LightCycler fluorophores unusable on the ABI PRISM 7700 (Nitsche *et al.*, 1999). The commercial form of the Bi-probe chemistry, called the ResonSense® probe (Defence Science and Technology Laboratory, United Kingdom; Lee *et al.*, 2002b), has overcome the platform-specific incompatibility possibly due to the substitution of SYBR Gold for SYBR Green I and the use of Cy5.5.

Single-fluorophore systems utilising a labelled oligoprobe or primer and FMCA to discriminate homozygous from heterozygous DNA without the need for FRET have also been described (Kurata *et al.*, 2001; Gundry *et al.*, 2003). The fluorophore is carefully chosen and positioned so that its emissions are quenched by proximity to a complementary guanine (Crockett and Wittwer, 2001). A commercial form, called the SimpleProbe, is used on the LightCycler and the LightTyper (Roche Diagnostics). This approach targets a single fluorescein-labelled hybridisation oligoprobe to polymorphism and produces an increased fluorescence when hybridised. Genotyping is performed by FMCA. The LightTyper is not a thermal cycler and as such this is not a real-time PCR system. The LightTyper is designed to rapidly characterise amplicon generated by PCR in a conventional thermal cycler. These systems suffer if used for the detection of nucleotide polymorphisms in a guanine-rich sequence.

Hairpin oligoprobes

Molecular beacons were the first fluorogenic hairpin oligoprobes described for real-time PCR applications. The hairpin oligoprobe's fluorogenic labels are called the fluorophore and quencher and are positioned at the termini of the oligoprobe (Figure 10.13). The most commonly used quencher, DABCYL (4-[4′-dimethylamino-phenylazo]-benzene), is an NFQ. The labels are held in close proximity by distal regions of homologous base pairing deliberately designed to create
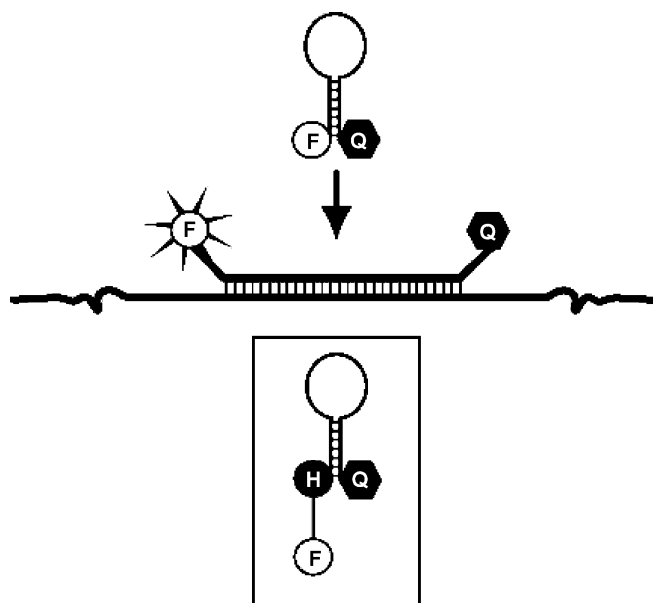
Figure 10.13. Function of the molecular beacon. Hybridisation of the loop section of the beacon to the target separates the fluorophore (F; open circle) and NFQ (Q; filled hexagon) allowing fluorescence. Data from this bimolecular system is collected during the annealing step of the PCR. Inset shows a wavelength-shifting hairpin oligoprobe incorporating a harvester molecule (H; filled circle).

a hairpin structure. The intimate proximity of the label molecules results in quenching either by FRET or direct energy transfer via a collisional mechanism (Tyagi *et al.*, 1998). In the presence of a sequence complementary to the molecular beacon's loop, the oligoprobe is shifted into an open configuration. The reporter is then removed from the quencher's influence and fluorescence can be detected (Tyagi and Kramer, 1996).

Wavelength-shifting hairpin oligoprobes are a recent improvement to the hairpin oligoprobe chemistry, making use of a second, "harvesting" fluorophore (Figure 10.13, inset). The harvester passes on excitation energy acquired from a blue light source as fluorescent energy in the far-red spectrum. A receptive "emitter" fluorophore can then be selected which uses the energy to produce light at characteristic wavelengths. This approach offers the potential for improved multiplex real-time PCR and nucleotide polymorphism analysis by increasing the number of emitters that can be excited using a single energising wavelength (Tyagi *et al.*, 2000). This is a useful workaround for instruments with a limited energising light source.

Recently, TMB have been added to the hairpin oligoprobe class of fluorogenic chemistry (Figure 10.14; Nutiu and Li, 2002). These highly complex oligoprobes combine a molecular beacon's hairpin with longer, unlabelled, single-stranded arms. Each arm is 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
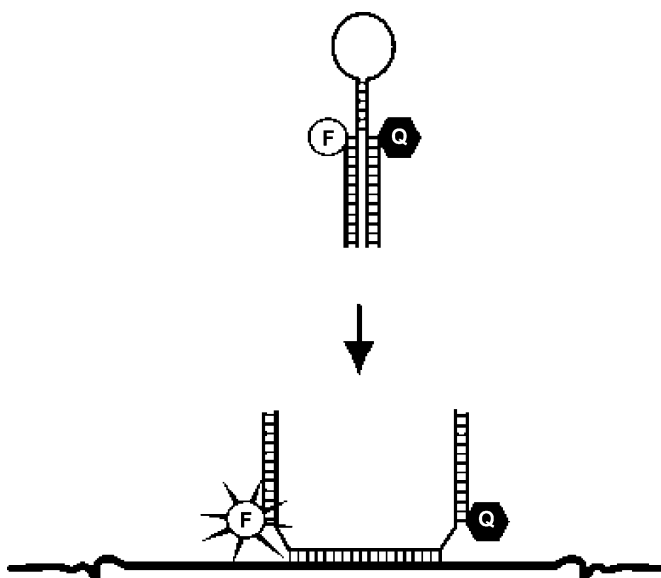
Figure 10.14. Function of the tripartite molecular beacon. The fluorophore (F; circle) is removed from the influence of the NFQ (Q; hexagon) upon binding to specific amplicon, which opens the hairpin and permits fluorescent emissions. Data are collected from this tetramolecular system during the annealing step of the PCR.

fluorescent when hybridised to the specific amplicon strand. However, for quenching to occur the arms must re-hybridise after the denaturation step. This is the only tetramolecular real-time PCR chemistry, so-called because it relies upon four intermolecular collisions to function correctly. The TMB chemistry may be subject to high background fluorescence in practical applications where genomic nucleic acids interfere with re-annealing.

Because the function of all the hairpin oligoprobes depends upon correct hybridisation of the stem, accurate design is crucial to their function and is considerably more challenging than for other oligoprobe chemistries.

Self-priming fluorogenic amplicon

The self-priming amplicon is similar in concept to the hairpin oligoprobe, except that the label(s) becomes irreversibly incorporated into the nascent amplicon. While these systems have only been described with a single primer, it is conceivable that both primers could be labelled in a similar way to provide a stronger, if more costly, fluorescent signal. The first contact between primer and template is a bimolecular event, but from then on the signalling is unimolecular. Intramolecular hybridisation is extremely fast and kinetically favourable since it does not rely upon the chance meeting of oligoprobe and amplicon in each cycle (Bustin, 2002). Fast cycling conditions appear to better suit the chemistries in this group (Thelwell *et al.*, 2000). Three approaches have been described: sunrise

primers (commercially called Amplifluor™ hairpin primers), scorpion primers (Nazarenko *et al.*, 1997) and the recently described Light Upon eXtension primers (LUX; Whitcombe *et al.*, 1999).

Sunrise primers consist of a 5′ fluorophore and a 3′ DABCYL NFQ (Figure 10.15). The labels are brought together by complementary sequences that create a stem when the sunrise primer is closed. A target-specific primer sequence is located at the 3′ terminus downstream of the NFQ. The sunrise primer's sequence is intended to be duplicated by the nascent complementary strand and during subsequent annealing and extension steps, the stem is destabilised, the two fluorophores are forced approximately 20 nt (70 Å) apart and the fluorophore can emit excitation

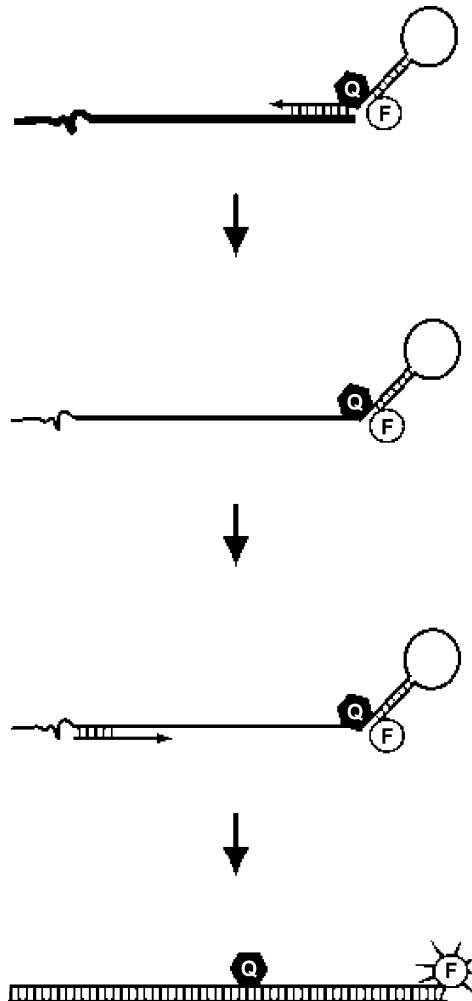**Figure 10.15.** Function of the sunrise primer. The fluorophore (F; circle) is separated from the NFQ (Q; hexagon) during disruption of the sunrise primer's hairpin structure and free to fluoresce. This disruption occurs during extension of a nascent complementary DNA strand and whenever dsDNA duplexes form during re-annealing. Data from this unimolecular system can be collected during the annealing step of the PCR.

energy (Nazarenko *et al.*, 1997). It is possible, however, that this sequence duplication could also occur during the formation of non-specific amplicon.

The scorpion primer is almost identical in design to the sunrise primer except for a hexethylene glycol molecule that blocks duplication of the signalling portion of the scorpion (Figure 10.16). In addition to the difference in structure, the function of the scorpion primer differs in that the 5′ region of the oligonucleotide is designed to hybridise to a complementary region within the nascent amplicon strand creating an intramolecular signalling system. This approach to signal generation separates the labels disrupting the hairpin and permitting fluorescence in the same way that hairpin oligoprobes function (Whitcombe *et al.*, 1999). The duplex scorpion primer is initially a trimolecular system requiring the primer, quencher and target amplicon to interact. However, once the primer is incorporated into the nascent strand the system reverts to
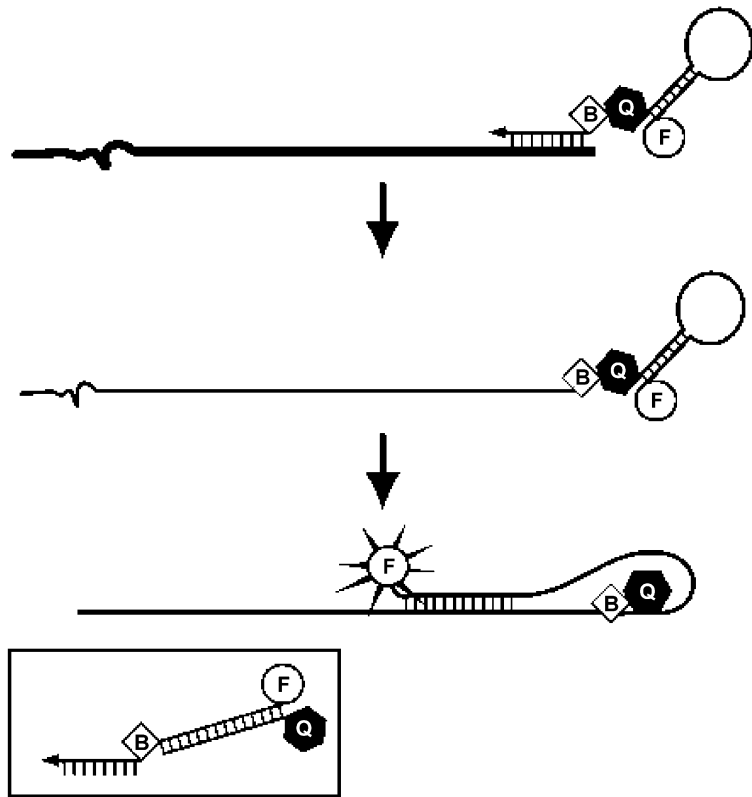
Figure 10.16. Function of the scorpion primer. The scorpion primer is blocked from being extended by a hexethylene glycol molecule (B; diamond) so that the hairpin can only be disrupted by specific hybridisation and not by the extension of a complementary amplicon strand as occurs for sunrise primers. The 5′ fluorophore (F; circle) is separated from a 3′ methyl red quencher (Q; hexagon) during self-hybridisation of the loop portion of the scorpion with a complementary region on the nascent amplicon strand. Inset shows a duplex scorpion. Data is collected from this unimolecular system during the annealing step of the PCR.

a bimolecular system which is simpler to synthesise and purify than the unimolecluar form. The stem-loop is exchanged for a separate, complementary oligonucleotide labelled with a quencher at the 5′ terminus. The additional oligonucleotide interacts with the primer element which is terminally labelled with the fluorophore (Figure 10.16, inset; Solinas *et al.*, 2001). When it exists as a duplex, the chemistry is quenched but becomes fluorescent after hybridisation to the longer specific amplicon strand. Interestingly, because the quencher is not part of the same molecule, brighter fluorescence can be achieved than for the unimolecular version where the labels are neither absolutely quenched, nor freely fluorescent. Scorpion primers can be used for nucleotide polymorphism detection by designing the placement of either the primer component or the probe component to cover the polymorphism (Whitcombe *et al.*, 1999; Thelwell *et al.*, 2000).

A recently described variation of the hairpin chemistry is the self-quenching hairpin oligonucleotide primer which has been commercially labelled as the LUX fluorogenic primer (Figure 10.17; Nazarenko *et al.*, 2002). This chemistry is non-fluorescent 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 fluorophore via a stretch of 5′ and 3′ complementary sequences. In the presence of the complementary target strand, a nascent strand is extended, which incorporates the LUX primer. The dsDNA opens the hairpin, permitting fluorescence from the fluorophore. This non-destructive chemistry is simple to design and use, relatively inexpensive and it does not require the inclusion of an additional oligoprobe. However, the presence of primer–dimer and
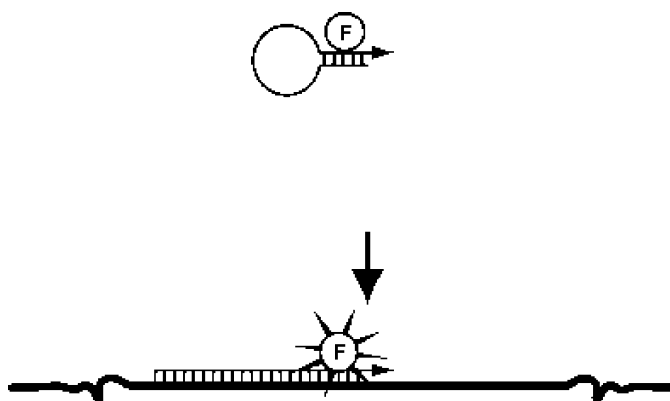
Figure 10.17. Function of the LUX primer. The LUX primer is labelled with a single fluorophore (F; circle) positioned next to a guanine nucleotide when the hairpin portion of the primer is intact. The G naturally quenches the fluorophore. In the presence of the specific target strand the primer hybridises, disrupts the hairpin and is extended. The fluorophore is now free to fluorescence and data can be collected from this unimolecular signalling system during the annealing or extension step of the PCR.

non-specific amplicon will, as for SYBR Green I and the sunrise primers, be displayed as a fluorogenic signal. The products can be discriminated using their differing T_D, however, there is no reliable way to account for their signal in quantitative real-time PCR.

A variant of these chemistries are the Angler® oligoprobes which closely resemble a Scorpion primer, except for the absence of a quenching moiety (Lee *et al.*, 2002b). Angler oligoprobes consist of a $3'$ specific primer sequence linked via a hexethylene glycol molecule to a $5'$, Cy5-labelled tail. The tail portion of the Angler is designed to self-hybridise to downstream sequences in the nascent strand producing a unimolecular signalling system. In contrast to the dual-labelled Scorpion, the Angler uses FRET between the terminal Cy5 and SYBR gold incorporated into the self-annealed duplex to produce a fluorescent signal.

Comparison of fluorogenic chemistries

When comparing the mechanism of signal generation by the different fluorescence systems, the most popular chemistries display unique quirks. The cyclical destruction of nuclease oligoprobes continues despite a plateau in amplicon accumulation whereas SYBR green I fluorescence generally increases non-specifically during later cycles even without template due to primer–dimer formation. HybProbe fluorescence begins to decrease as the rate of collision between the growing numbers of complementary amplicon strands increases resulting in a phenomenon called the "hook effect" (Figure 10.3). At this stage, the formation of dsDNA is favoured over the hybridisation of oligoprobe to its target DNA strand, adversely affecting total fluorescence. The possibility exists that some linear oligoprobes are consumed by sequence-related nuclease activity and it may also contribute to the hook effect as has been reported for HybProbes (Wilhelm *et al.*, 2001b; Wittwer *et al.*, 1997a; Lyamichev *et al.*, 1993).

◆◆◆◆◆◆ MICROBIAL GENOTYPING

Although nucleotide sequencing is the gold standard for in-depth characterisation of nucleic acids, it is a relatively lengthy process. The development of real-time PCR has partially addressed this by providing a tool capable of rapid detection of characterised nucleotide polymorphisms.

The non-destructive fluorescent chemistries are mostly intact at the end of the PCR and can be used to indirectly genotype a microbial template by characterising its amplicon (Bustin, 2000). Careful control of ionic strength, amplicon size and cooling and melting rates can improve the resolution of experimental data collected from FMCA. FMCA can also be used with a single labelled oligonucleotide (Gundry *et al.*, 2003). The SYBR green I and HybProbe chemistries are most commonly used

to perform these analyses; however, the double-stranded and Light-up oligoprobes as well as the HyBeacons should be capable of functioning in this role. Other chemistries, such as the TaqMan and Eclipse oligoprobes and hairpin oligonucleotides and primers, require two sets of oligoprobes to differentiate a wild-type from an altered sequence. While this is a perfectly legitimate and functional approach for genotyping by real-time PCR, the extra fluorogenic oligonucleotides increase the overall cost and complexity of the assay. However, this approach does permit the creation of a dedicated mutation detection assay which will often perform more reliably than a single assay intended to detect both the wild-type and mutated sequences. Unfortunately, the use of two sets of fluorophores reduces the number of related but different microbes that can be discriminated in a single vessel, since two fluorophores must be assigned to analyse each nucleotide variant. The technique of FMCA has proven popular for the rapid diagnosis of human genetic disorders and has advanced the detection of multiple targets by real-time PCR. It should again be noted that genotyping two sequences by FMCA requires that the amount of one amplified sequence is less than 10-fold above the amount of the other sequence; otherwise the more common sequence will overpower detection of the more rare sequence.

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 alternate configuration. Therefore, hairpin oligonucleotides are more specific than the commonly used linear oligoprobes making hairpin oligonucleotides good candidates for detecting nucleotide changes, albeit more technically demanding to design (Tyagi *et al.*, 1998). Despite the fact that hybridisation does not reach equilibrium using rapid ramp rates, the apparent T_M values deduced from FMCA are both reproducible and characteristic of a given probe and target duplex (Gundry *et al.*, 1999). Importantly, different nucleotide changes destabilise hybridisation to differing degrees and this can be incorporated into the design of oligoprobes for genotyping assays to control the extent of discrimination between the melt peak temperatures. The least destabilising mismatches involve a change to a G on one of the strands (G:T, G:A, and G:G), whereas the most destabilising include a change to a C (C:C, C:A and C:T) (Bernard *et al.*, 1998).

Because real-time PCR genotyping data is obtained at the completion of the PCR amplification phase, it is an end-point analysis, although the reaction vessel remains unopened throughout the process. 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 duplex destabilisation incurred as a result of nucleotide changes. As mentioned earlier, the non-specific chemistries reflect these changes in the context of the entire dsDNA amplicon.
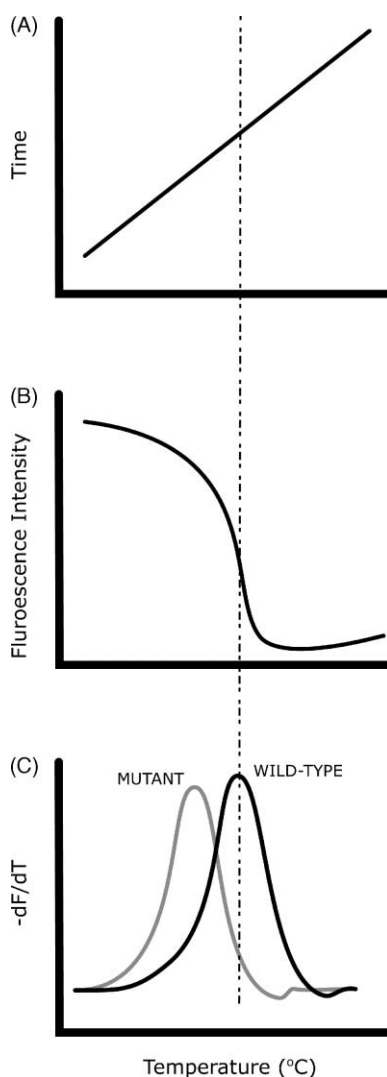
Figure 10.18. Fluorescence melting curve analysis (FMCA). At the completion of a real-time PCR using a fluorogenic chemistry, the reaction is rapidly heated and then cooled to a temperature below the expected T_D of the dsDNA or T_M of the oligoprobe(s). It is then heated to 85°C or more at a fraction of a degree per second (A). During heating, the raw data representing the emissions of a relevant fluorophore are constantly acquired (B). Software calculates the negative derivative of the fluorescence with temperature which is plotted against temperature to produce a melt peak indicative of the T_D of the dsDNA, or the T_M of the oligoprobe-target melting transition (dashed line and black peak; C). When sequence differences exist, the T_D or T_M is reduced (grey peak) due to the lower stability of heteroduplexes and the extent of the resulting temperature shift is used diagnostically to characterise an amplified template. Generally speaking, a ramp rate of 0.2°C/s permits clear discrimination between different genotypes. However, variation of the ramp rate can be helpful if unsatisfactory results are generated. Hybridisation can be enhanced by slowing the ramp rate whereas an accelerated ramp rate can help remove stable secondary structures at the oligoprobe hybridisation region resulting in sharper melt peaks.

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 difference. 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 and plotting that data against temperature (Figure 10.18).

◆◆◆◆◆◆ NUCLEIC ACID QUANTITATION

Although the terminology is often confused, real-time PCR does not inherently imply quantitative PCR. To determine the amount of template present in a sample, care must be taken to include the correct control system. External controls or "standards" are used for calculating the amount of template present in a patient sample and are amplified in a separate vessel to the unknown target (Figure 10.19). Internal controls are amplified in the same vessel as the unknown target, frequently indicating the occurrence of false negative reactions and examining the ability to amplify from a preparation of nucleic acids (Niesters, 2002). Certainly, the reliability of quantitative PCR methods is intimately associated with the choice and quality of the assay controls (Celi *et al.*, 2000; Alexandre *et al.*, 1998).

No matter what controls are used to generate quantitative data or monitor successful amplification, it is imperative to accurately determine their concentration and to ensure that internal controls are added at suitable levels in order to prevent extreme competition for reagents with the wild-type template (Zimmermann and Manhalter, 1996; Brightwell *et al.*, 1998). A spectrometer alone is inadequate for quantifying a control molecule; however, in combination with experimental and statistical analysis, the reliability of the data is greatly enhanced (Glasel, 1995; Bagnarelli *et al.*, 1995; Wang and Spadoro, 1998; Rodrigo *et al.*, 1997; Taswell, 1981; Sykes *et al.*, 1998). Finally, one must remember that the results of quantitation using a 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. Standardisation such as this will ensure comparability between assay results and testing locations (Niesters, 2001).

The ability to quantify nucleic acids is predominantly used in the field of gene expression or transcriptome analysis (PhorTech, 2003). For this application, primers are often carefully designed to span exon–intron boundaries or cross overly large expanses of intron to avoid co-amplification of genomic DNA (Yin *et al.*, 2001). In our hands the enhanced specificity of an oligoprobe format is preferred to the use of non-specific chemistries for quantitation.

Another area suited to sensitive, specific quantitation with the ability to genotype a target is the detection of minimal residual disease in malignancy. The role of real-time PCR for the investigation of lymphoma

$$y = mx + b \qquad ..i$$
$$x = (y - b) / m \qquad ..ii$$
$$Unk = (C_T - y\text{-intercept}) / slope \qquad ..iii$$
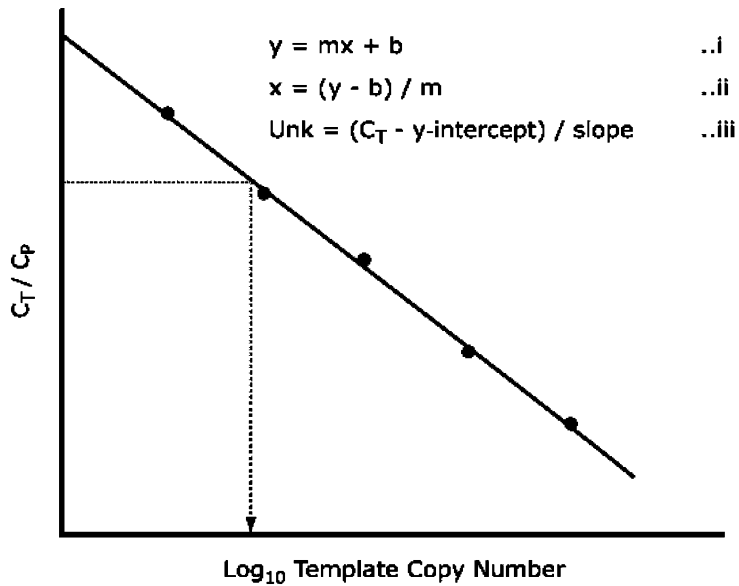
Figure 10.19. External standard curve for quantitation. Threshold cycle or crossing point data collected from an amplified titration of standard plotted against the concentration of each template. Through interpolation, C_T/C_P values for unknowns (Unk) permit the calculation of the starting template concentrations (dashed line). This is performed by rearranging the equation for the linear regression line (i), to solve for the *x*-axis value of interest, (ii). When the relevant values are substituted (iii), the concentration of template in each unknown can be calculated.

and leukaemia patients is expanding. This role examines and quantifies the number and genetic profile of malignant cells remaining in a clinically well patient as an indicator of response to therapy or to identify those patients at high risk of disease relapse (Sharp and Chan, 1999; Liu Yin, 2002).

External Control Templates

Standards used to monitor or enhance the power of PCR are most commonly created by cloning an amplicon, a portion of the target organism's genome or simply using the purified amplicon itself as the template (Borson *et al.*, 1998). Because these molecules are amplified separately and because they are not inherently present within the sample matrix, they are called exogenous or external controls. These form the basis of external standard curves, which are created from the data produced by the individual amplification of a dilution series of control template (Figure 10.3). The concentration of an unknown, which is amplified in the same assay but in a separate vessel, can then be interpolated from the standard curve (Figure 10.19). While the external standard curve is the more commonly described approach for quantitative PCR, it frequently suffers from uncontrolled and unmonitored inter-vessel variations. Some

real-time PCR systems have partially overcome this problem by including the ability 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). This reference is present in the reaction buffer and is used to indicate volume variations and non-specific fluorescence quenching. 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 $RQ+$ for each sample, resulting in the ΔRQ value that indicates the magnitude of the reference signal generated for the given PCR (Gelmini *et al.*, 1997).

The simplest mathematical representation of PCR amplification is described by the Equation:

$$N = N_0(E + 1)^C$$

where N is the number of amplified molecules, N_0 the initial number of template molecules, E the amplification efficiency and C the number of amplification cycles.

This equation is essential to obtaining accurate data from real-time PCR and is usually considered to apply to each reaction in an experimental run. However, the efficiency of the PCR is commonly calculated from the amplification of a standard curve, assuming that each unknown reaction will amplify with equivalent efficiency. Furthermore, the amount of amplicon in each reaction at the C_T is assumed to be equal because the fluorescence is the same (Figure 10.3). Nonetheless, by adopting these assumptions and deducing the linear regression line of best fit through the C_T values from a standard curve we can determine the reaction efficiency from the slope given by the following Equation:

$$E = 10^{-\text{Slope}} - 1$$

Internal Control Templates

The use of an internal control molecule has been described in the earliest of PCR experiments and is considered essential to increase assay quality (Reiss and Rutz, 1999) and as a competitive template for conventional quantitative PCR (Chehab *et al.*, 1987; Rosenstraus *et al.*, 1998). When such a control is added prior to template purification (extraction control) or amplification (amplification control), it is called an exogenous internal control since it does not occur naturally within the sample matrix, but it is co-amplified within the same reaction. Ideally the exogenous internal control template should hybridise to the same primers and have an identical amplification efficiency to the template under investigation (Zimmermann and Manhalter, 1996). The control should also contain a discriminating feature such as a change in its length (Orlando *et al.*, 1998; Möller and Jansson, 1997; Brightwell *et al.*, 1998; Hall *et al.*, 1998, Celi *et al.*, 2000) or more commonly in today's oligoprobe-based methods, a change

in the sequence of the target (Alexandre *et al.*, 1998; Tarnuzzer *et al.*, 1996; Celi *et al.*, 2000; Natarajan *et al.*, 1994; Aberham *et al.*, 2001; Rosenstraus *et al.*, 1998; Kearns *et al.*, 2001b; Stöcher *et al.*, 2002; Gruber *et al.*, 2001). However, the internal control should not significantly interfere with the detection of small quantities of the template under investigation; therefore, its use must be carefully evaluated. Internal control templates that bind different primers or have different amplification efficiencies are still useful as standards for semi-quantitative PCR or relative quantitation.

An endogenous internal control occurs naturally within the specimen matrix and is therefore co-amplified with the specific target. House-keeping genes are a common example that has been successfully used to quantify gene expression by RT-PCR and monitor template integrity after its purification (Chehab *et al.*, 1987). When housekeeping genes are used for the quantitation of RNA it is essential that they are minimally regulated and exhibit a constant and cell cycle-independent basal level of transcription (Selvey *et al.*, 2001). Studies have shown that an 18S rRNA target successfully meets the desired criteria for some applications (Selvey *et al.*, 2001; Thellin *et al.*, 1999). This is not the case for some commonly used genes including β-actin; unfortunately their use is widespread, especially for transcript analyses (Härtel *et al.*, 1999). To ensure the suitability of the housekeeping gene, a panel of candidate targets should be examined under the desired experimental conditions and the extent of change in the C_T used to indicate the stability of target expression (Löseke *et al.*, 2003).

We have found several promising candidate targets during a systematic quantitative study of the expression levels of 13 genes in 16 different tissues (Radonic *et al.*, 2004). As expected, no single gene was consistently expressed among all the tissues investigated. However, the RNA polymerase gene displayed the lowest variation over all tissues, even during mitogenic stimulation of a T-cell line. RNA polymerase mRNA encodes the principle enzyme used in mRNA transcription and is therefore part of a self-regulating cycle. It can be assumed that this gene is expressed steadily and independent of the cell state.

Real-time PCR assays that lack the capacity to correct for vessel-to-vessel variations such as those caused by variation in reverse transcription efficiency, cell number, nucleic acid isolation technique and PCR efficiency are more appropriately described as being semi-quantitative. To this day it is rare to find well-controlled real-time PCR assays among those in the literature. That will hopefully change as pressure to publish comprehensively optimised and validated assays is brought to bear by the editorial staff and peer reviewers of scientific journals (Hoorfar *et al.*, 2003).

Relative versus Absolute Quantitation

The amount of template in a sample can be described either relatively or absolutely. Relative quantitation is the simpler approach, which, as the name suggests, describes differences 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. This approach has proven particularly popular for gene expression studies and can be conveniently employed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Absolute quantitation is more demanding to perform but states the exact number of nucleic acid targets present in the specimen in relation to a specific unit and it is therefore easier to compare data between different assays and laboratories (Freeman *et al.*, 1999; Pfaffl *et al.*, 2002). Absolute quantitation may be necessary when there are no sequential specimens to demonstrate a relative change in template load, or when no accurately standardised reference reagent or suitable endogenous control is available.

A highly accurate approach used for absolute quantitation by conventional PCR utilises competitive co-amplification of one or more dilutions of an internal control template of known concentration with a wild-type target nucleic acid of unknown concentration (Becker-Andre and Hahlbrock, 1989; Clementi *et al.*, 1995; Gilliland *et al.*, 1990; Siebert and Larrick, 1992). However, conventional competitive quantitation is technically demanding and time consuming, requiring significant development and optimisation compared to quantitation by real-time PCR. Thus, it is not suited to the quick decision making required within research and clinical environments (Locatelli *et al.*, 2000; Wall and Edwards, 2002; Tanaka *et al.*, 2000b). The existence of software with the ability to calculate the concentration of an unknown by comparing real-time PCR signals generated from a co-amplified target and internal control is rare but improving (Pfaffl *et al.*, 2002). In addition, new or improved mathematical interpretations are appearing which aim to make quantitation more reliable and simpler (Liu and Saint, 2002; Rutledge and Côte, 2003).

Improved Quantitation Using Real-time PCR

Common microbiological methods to quantify an organism have traditionally required culture-based methods. While these methods are often considered the gold standard and are the only way to obtain data on microbial viability and infectivity, they are not without significant problems of their own. These problems are caused by variation in the method of inoculation, the choice of target cell, the type of culture medium, the sensitivity of the microbe to transport and storage conditions and the conditions of culture (Luria and Darnell, 1967). One must remember that titres obtained from culture only hold true under the specific conditions of that assay, making the results less portable than those obtained from nucleic acid quantitation by well-developed real-time PCR assays.

We have used conventional competitive PCR to obtain quantitative data with promising results (Mackay *et al.*, 2001, 2003a). However, these approaches are laborious to develop and perform. The wide dynamic range of real-time PCR encompassing at least eight $\log_{10}$ copies of nucleic

acid template is a significant benefit for microbial load determination (Ishiguro *et al.*, 1995; Kimura *et al.*, 1999; Najioullah *et al.*, 2001; Ryncarz *et al.*, 1999; Monopoeho *et al.*, 2000; Alexandersen *et al.*, 2001; Abe *et al.*, 1999; Locatelli *et al.*, 2000; Gruber *et al.*, 2001; Brechtbuehl *et al.*, 2001; Moody *et al.*, 2000). This broad dynamic range obviates the need to dilute an amplicon before detecting it or the repetition of an assay using a diluted template because a preliminary result falls above the upper limits of the detection assay. Both these problems occur when using conventional end-point PCR assays for quantitation because their detection systems cannot encompass the products of high template loads whilst maintaining adequate sensitivity (Weinberger *et al.*, 2000; Schaade *et al.*, 2000; Brechtbuehl *et al.*, 2001; Kawai *et al.*, 1999). 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 or, with careful manipulation, to quantify expression from a single cell (Ryncarz *et al.*, 1999; Liss, 2002).

Real-time PCR is also a particularly attractive alternative to conventional PCR for the study of microbial load. We and others have found the consistently low inter-assay and intra-assay variability especially useful compared to other assays (Schutten *et al.*, 2000; Nitsche *et al.*, 2000; Locatelli *et al.*, 2000). Real-time PCR also has equivalent or improved sensitivity compared to microbial culture, or conventional single-round and nested PCR (Kearns *et al.*, 2001c; Clarke *et al.*, 2002; Kupferschmidt *et al.*, 2001; Kennedy *et al.*, 1997b; Locatelli *et al.*, 2000; Capone *et al.*, 2001; Leutenegger *et al.*, 1999; Smith *et al.*, 2001; Monopoeho *et al.*, 2000; van Elden *et al.*, 2001; Lanciotti *et al.*, 2000). It 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 (Capone *et al.*, 2001).

◆◆◆◆◆◆ MULTIPLEX REAL-TIME PCR

Conventional multiplex PCR applies a number of primer sets to potentially amplify multiple templates within a single reaction vessel (Chamberlain *et al.*, 1988; Burgart *et al.*, 1992). However, its adaptation to real-time PCR has clouded the traditional terminology. Multiplex real-time PCR more commonly describes the use of multiple fluorogenic oligoprobes for the discrimination of amplicons which have been produced by one or more primer pairs in a single vessel. The transfer of this technique from conventional PCR protocols has proven problematic because of the limited number of fluorophores available and the closed nature of real-time PCR which prohibits the monitoring of specificity made possible by size determination using agarose gel electrophoresis (Lee *et al.*, 1993). The commonly used monochromatic energising light source and the application of narrow bandpass filters on some real-time PCR instruments have limited their ability to perform multiplex PCR. Although excitation by a single wavelength produces bright emissions from an appropriate fluorophore, it restricts the number of fluorophores

that can be successfully combined and discriminated in a single vessel (Tyagi *et al.*, 2000). The discovery and application of non-fluorescent quenching labels has made available some regions of the spectrum that were previously occupied by the emissions of early quenchers. This development has permitted a slight increase in the number of spectrally discernable oligoprobes per reaction.

Recent improvements to the design of hairpin primers and hairpin and nuclease oligoprobes, as well as novel combinations of fluorophores such as the Bi-probe and Light-up probes, have promised alternatives that should permit the discrimination of a greater variety of amplicons per reaction vessel.

Currently, the number of fluorophores that can be combined and clearly distinguished is extremely limited when compared to the capability of conventional multiplex PCR to discriminate amplicon. Some real-time PCR platforms have incorporated multiple light-emitting diodes to span the entire visible spectrum, or a tungsten light source, which emits light over a broad range of wavelengths permitting the excitation of a wide range of fluorophores. The inclusion of high-quality optical filters to clarify both the excitation and emission energies makes possible the use of a wider range of real-time PCR detection chemistries on one machine. Nonetheless, these improvements have to date permitted the multiplexing of only four different colours, of which one colour is ideally set aside for an internal control to monitor inhibition and one may be required for a passive reference. Some real-time PCR designs have astutely made use of single or multiple nucleotide changes between similar templates to discriminate the amplicon by T_M using FMCA and thus avoid the need for multiple fluorophores (Espy *et al.*, 2000a,b; Boivin *et al.*, 2004; Schalasta *et al.*, 2000a; Kearns *et al.*, 2001c; Loparev *et al.*, 2000; Read *et al.*, 2001; Whiley *et al.*, 2001). This approach has been common for the detection of human genetic diseases where as many as 27 possible nucleotide substitutions have been detected using only one or two fluorophores (Schütz *et al.*, 2000; Lay and Wittwer, 1997; Herrmann *et al.*, 2000; Lee *et al.*, 1999a; Gundry *et al.*, 1999; Bernard and Wittwer, 2000; Elenitoba *et al.*, 2001).

To date, only a handful of assays have been described in the literature that truly multiplex more than two fluorophores. Few of these duplexed assays have been applied to the diagnosis of infectious disease and those that have are frequently used to detect subtle changes within a single species rather than a number of different species. It is important to remember that the ultimate benefits to be gained from multiplex PCR derive from its use of minimal specimen to detect the maximum number of targets. Some approaches cannot technically be considered real-time homogeneous assays, because they require interruption of the procedure to transfer template, detect fluorescence using a separate end-point analysis or perform the assays in separate reaction vessels but within the same run (Stocher *et al.*, 2003). The best microbial multiplex real-time PCR can discriminate between four retroviral target sequences (Vet *et al.*, 1999); however, we and others have found conventional multiplex PCR using end-point detection

capable of easily discriminating five or more different amplicons (Mackay *et al.*, 2003a; Quereda *et al.*, 2000). This discrepancy continues to highlight the greater flexibility of conventional PCR for true multiplex PCR assays (Kehl *et al.*, 2001; Echevarria *et al.*, 1998; Henegariu *et al.*, 1997; Weigl *et al.*, 2000; Stockton *et al.*, 1998).

Assays capable of detecting several viral genomes include those using the non-specific label, SYBR green I, to detect herpes simplex viruses (HSV), varicella zoster virus (VZV) or cytomegalovirus (CMV) in separate tubes (Nicoll *et al.*, 2001), or by adaptation of a conventional multiplex PCR, to identify HSV-1 and HSV-2, VZV and enteroviruses within a single capillary by applying FMCA (Read *et al.*, 2001).

Future development of novel chemistries and improved real-time PCR instrumentation and software will hopefully enhance our ability to perform truly multiplexed real-time PCR assays. Meanwhile, the promise of multiplex real-time PCR applications remains tantalisingly out of reach.

◆◆◆◆◆◆ APPLICATION OF REAL-TIME PCR TO MICROBIOLOGY

Microbiology has been a significant driving force for the development of real-time PCR technology. The resulting benefits to microbial studies have already been discussed in a number of elegant reviews (Niesters, 2001; Mackay *et al.*, 2002; Cockerill and Smith, 2002; Versalovic and Lupski, 2002; Niesters, 2002; Bretagne, 2003). The final sections of this chapter will update the reader on the use of real-time PCR for the study of viruses, bacteria, fungi, parasites and protozoans as well as some specialist areas that have been significantly enhanced by the technology.

Viruses

In microbiology, the application of real-time PCR has had the most dramatic impact upon virology where it has provided an alternative to assays which either use morbidity and mortality as an indicator of disease progression, or qualitatively investigate the role of viruses in a range of human diseases (Kato *et al.*, 2000). Also, epidemiological studies of co-infections have been improved by the use of duplex real-time PCR (Zerr *et al.*, 2000; Furuta *et al.*, 2001; Kearns *et al.*, 2001c).

Real-time PCR has been used as a tool to complement conventional research techniques when investigating direct and indirect links between viral infection and chronic conditions such as sarcoma (Kennedy *et al.*, 1997a,b, 1998a; O'Leary *et al.*, 2000a,b), carcinoma (Lo *et al.*, 1999; Capone *et al.*, 2001), cervical intraepithelial neoplasia (Josefsson *et al.*, 1999; Lefevre *et al.*, 2003; Swan *et al.*, 1997; Lanham *et al.*, 2001) and lymphoproliferative disorders (MacKenzie *et al.*, 2001; Jabs *et al.*, 2001).

More commonly, real-time PCR studies simply describe the presence of viruses in their host with a long-term view of improving clinical management of the affected patients. Viruses studied to date include the bunyaviruses (Stram *et al.*, 2004), caliciviruses (Song *et al.*, 1997), flaviviruses (Laue *et al.*, 1999; Kilpatrick *et al.*, 1996; White *et al.*, 2002; Callahan *et al.*, 2001; Ratge *et al.*, 2002; Ishiguro *et al.*, 1995; Komurian-Pradel *et al.*, 2001; Lanciotti *et al.*, 2000; Beames *et al.*, 2000), hepadnaviruses (Weinberger *et al.*, 2000; Chen *et al.*, 2001; Cane *et al.*, 1999; Brechtbuehl *et al.*, 2001), herpesviruses (Nitsche *et al.*, 2000; Takaya *et al.*, 1996; Song *et al.*, 2002; Whiley *et al.*, 2003b; Fan and Gulley, 2001; Schalasta *et al.*, 2000b; Kearns *et al.*, 2001a; Stevens *et al.*, 2002; Loparev *et al.*, 2000; Gallagher *et al.*, 1999; Kennedy *et al.*, 1997b; Fernandez *et al.*, 2002; Biggar *et al.*, 2000; Tanaka *et al.*, 2000b; Locatelli *et al.*, 2000; Ohyashiki *et al.*, 2000; Kearns *et al.*, 2001c; Lallemand *et al.*, 2000; White and Campbell, 2000; Najioullah *et al.*, 2001; Schaade *et al.*, 2000; Capone *et al.*, 2001; Lo *et al.*, 1999; Niesters *et al.*, 2000; Kimura *et al.*, 1999; Ryncarz *et al.*, 1999; Peter and Sevall, 2001; Hawrami and Breur, 1999; Furuta *et al.*, 2001), orthomyxoviruses (van Elden *et al.*, 2001; Ellis and Zambon, 2002), papovaviruses (Jordens *et al.*, 2000; Lefevre *et al.*, 2003; Biel *et al.*, 2000; Whiley *et al.*, 2001), paramyxoviruses (Smith *et al.*, 2001; Daniels *et al.*, 2001; Côte *et al.*, 2003; Mackay *et al.*, 2003b; Whiley *et al.*, 2002b; Aldous *et al.*, 2001), parvoviruses (Gruber *et al.*, 2001), pestiviruses (Vilcek and Paton, 2000), picornaviruses (Alexandersen *et al.*, 2001; Kares *et al.*, 2004; Lai *et al.*, 2003; Hu *et al.*, 2003; Reid *et al.*, 2004; Monpoeho *et al.*, 2002; Nijhuis *et al.*, 2002; Corless *et al.*, 2002; Watkins-Reidel *et al.*, 2002; Verstrepen *et al.*, 2001, 2002; Monopoeho *et al.*, 2000), polydnaviruses (Chen *et al.*, 2003), poxviruses (Espy *et al.*, 2002; Ibrahim *et al.*, 1997, 1998; Shaw *et al.*, 1995), reoviruses (Pang, 2004), retroviruses (Lewin *et al.*, 1999; Lew *et al.*, 2004a; Argaw *et al.*, 2002; Schutten *et al.*, 2000; Choo *et al.*, 2000; Klein *et al.*, 1999; Leutenegger *et al.*, 1999), rhabdoviruses (Smith *et al.*, 2002; Hughes *et al.*, 2004) and TT virus (Iriyama *et al.*, 1999).

Less commonly, studies of the pathogenic effects of viruses on the biology of specific cellular populations has provided a snapshot of the mechanism of disease progression as occurs during hepatitis C virus induced chronic liver disease (Shaw *et al.*, 1995).

Over the last decade, conventional quantitative PCR has repeatedly demonstrated that the determination of viral load is a useful marker of disease progression and a valuable way to monitor the efficacy of antiviral compounds (Holodniy *et al.*, 1991; Held *et al.*, 2000; Roberts *et al.*, 1998; Rollag *et al.*, 1998; Kaneko *et al.*, 1992; Clementi *et al.*, 1995; Clementi, 2000; Menzo *et al.*, 1992). Quantitative real-time PCR has similarly examined the interaction between virus and host and monitored changes in viral load resulting from antiviral therapy ultimately impacting on the treatment regimen selected (Nitsche *et al.*, 1999; Clementi, 2000; Limaye *et al.*, 2000). Because disease severity and viral load are linked, microbial quantitation by real-time PCR has proven beneficial when studying the impact of viral reactivation, persistence or isolated gene expression on the progression of disease (Ohyashiki *et al.*, 2000; Chen *et al.*, 2003; Kearns *et al.*, 2001c; Furuta *et al.*, 2001; Limaye *et al.*, 2001; Tanaka *et al.*, 2000a,b; Lallemand *et al.*,

2000; Laue *et al.*, 1999; Kimura *et al.*, 1999; Chang *et al.*, 1999; Lo *et al.*, 1999; Hawrami and Breur, 1999; Hoshino *et al.*, 2000; Nitsche *et al.*, 2000; Machida *et al.*, 2000; Limaye *et al.*, 2001; Najioullah *et al.*, 2001; Gault *et al.*, 2001). Altered microbial tropism or replication and the effect of these changes on the host cell can also be followed using real-time PCR (Kennedy *et al.*, 1998a, 1999a,b). Molecular assays are increasingly being used to augment or replace traditional assays. Two examples are the use of real-time PCR to calculate bacteriophage titer more accurately and over a broader dynamic range than plaque assays and the indirect determination of neutralising antibody titer to chicken anemia virus in chickens by quantifying viral load (Edelman and Barletta, 2003; van Santen *et al.*, 2004).

The speed and flexibility of real-time PCR has proven useful to commercial interests that require exquisite sensitivity to screen for microbial contamination within large-scale reagent preparations produced from eukaryotic expression systems or among livestock used for the production of food (Brorson *et al.*, 2001; Pinzani *et al.*, 2004; Stram *et al.*, 2004; Reid *et al.*, 2004; Lew *et al.*, 2004b; van Santen *et al.*, 2004; Rudi *et al.*, 2002; de Wit *et al.*, 2000). Additionally, highly sensitive assays are proving invaluable for the thorough assessment of viral gene therapy vectors prior to their use in clinical trials. Nuclease oligoprobes have been most commonly used for these studies, which assess the biodistribution, function and purity of the novel "drug" preparations (Gerard *et al.*, 1996; Suzuki *et al.*, 2003; Barzon *et al.*, 2003; Rohr *et al.*, 2002; Josefsson *et al.*, 2000; Hackett *et al.*, 2000; Sanburn and Cornetta, 1999; Choo *et al.*, 2000; Scherr *et al.*, 2001).

Likewise, the study of new and emerging viruses has embraced 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 (Lanciotti and Kerst, 2001; Mackay *et al.*, 2003b; Smith *et al.*, 2001, 2002; Lanciotti *et al.*, 2000; Halpin *et al.*, 2000; Gibb *et al.*, 2001a,b).

The severe acute respiratory syndrome (SARS) arose in early 2003 in China and the causative agent was rapidly identified (Peiris *et al.*, 2003; Poutanen *et al.*, 2003; Tsang *et al.*, 2003; WHO, 2003). As soon as the first stretches of sequence were known, real-time PCR assays provided the potential for fast and reliable diagnoses and epidemiological studies (Drosten *et al.*, 2003; Emery *et al.*, 2004; Zhai *et al.*, 2004; Ruan *et al.*, 2003; Ng *et al.*, 2004). These assays are performed either as one-step or two-step RT-PCR assays. At the time of writing 48 complete SARS coronavirus (SARS-CoV) genomic sequences existed on GenBank. All these sequences are closely related. However, as with all RNA virus genomes, the coronaviruses display a tendency to vary with time and location (Wood, 2003). We recently described an approach to maintain the reliability of SARS coronavirus molecular assays. In this approach, three independent real-time PCR assays, based on the 5′ nuclease format, were established to be performed under identical reaction conditions. All three assays are located in different, yet highly conserved regions of the known SARS-CoV genomes separated by approximately

9000 nucleotides. Because of this distance, under the chosen reaction conditions the primers used in one assay cannot react with primers of another assay performed in the same tube. The fluorescence signals produced by all three individual assays are indistinguishable; however, for diagnostic purposes it is not necessary to assign the signals to a certain amplicon. We were able to demonstrate that the sensitivity was maintained by the simultaneous amplification of three amplicons. The strength of this approach is that all three assays would need to be adversely affected by nucleotide change for the diagnostic system to fail, a virtually impossible occurrence. We believe that the amplification of more than one sequence stretch of the pathogen of interest is a useful technique to increase the reliability of diagnosis, especially for new and emerging microbes.

Nonetheless, the absolute reliance of PCR upon fully representative characterised genome sequences is highlighted in this area of microbial study. The early assay developer may suffer setbacks due to a paucity of sequence data representing all viral variants. We found this to be a problem when developing a 5′ nuclease assay for human metapneumovirus (Mackay *et al.*, 2003b). At the time of development the majority of viral sequences present on GenBank represented what was later to become known as the Type A virus – a bias which was reflected by the new assay.

Bacteria

Rapid real-time PCR assays provide significant benefit to the diagnosis of a bacterially infected host. The results can quickly inform the clinician, allowing a more specific and timely application of antibiotics, which are far more than antiviral therapies. This speed can limit the potential for toxicity caused by shotgun treatment regimens, reduce the duration of a hospital stay and prevent the improper use of antibiotics, minimising the potential for resistant bacterial strains to emerge.

Once again, elegant applications of real-time PCR can augment or even replace traditional culture and histochemical assays as was seen with the creation of a molecular assay capable of classifying bacteria in the same way as a Gram stain (Klaschik *et al.*, 2002). However, particular bacterial species are the more frequent focus of real-time PCR assays especially when slow culture times can be replaced by rapid and specific gene detection. *Leptospira* genospecies, *Mycobacterium* and *Propionibacterium* species, *Chlamydia* species, *Legionella pneumophila* and *Listeria monocytogenes* have all been successfully detected and in some cases quantified using real-time PCR assays (Woo *et al.*, 1997; DeGraves *et al.*, 2003; Huang *et al.*, 2001; Rudi *et al.*, 2002; Nogva *et al.*, 2000; Desjardin *et al.*, 1998; Bassler *et al.*, 1995; Miller *et al.*, 2002; O'Mahony and Hill, 2002; Torres *et al.*, 2000; Eishi *et al.*, 2002; Kraus *et al.*, 2001; de Viedma *et al.*, 2002; Li *et al.*, 2000; Creelan and McCullough, 2000; Hayden *et al.*, 2001; Ballard *et al.*, 2000; Wellinghausen *et al.*, 2001; Reischl *et al.*, 2002; Lunge *et al.*, 2002). Real-time RT-PCR has proven useful for quantitation of bacterial

transcripts in response to infection or the application of specific metabolic intermediates; however, these assays do not always agree with conventional methods of microbial detection, indicating the difference between detecting a live organism by culture and a dead organism by fluorescence or DNA detection (Goerke *et al.*, 2001; Corbella and Puyet, 2003; Desjardin *et al.*, 1998).

The detection of *Neisseria gonorrhoeae* has benefited from real-time PCR, particularly when used as a confirmatory test for commercial assays (Whiley *et al.*, 2002a). This example again demonstrates the need for care when choosing a PCR target, especially when that target exists on a plasmid which is exchanged among other bacteria, potentially providing confusing diagnostic results. *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* are the major pathogens causing bacterial meningitis and the introduction of diagnostic real-time PCR has proven to be a powerful tool that we and others have quickly developed and deployed for the rapid discrimination of circulating pathogens (Corless *et al.*, 2001; Whiley *et al.*, 2003a; Probert *et al.*, 2002; Mothershed *et al.*, 2004).

The detection and monitoring of antibiotic resistance among clinical isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Helicobacter pylori*, *Enterococcus faecalis* and *Enterococcus faecium* has benefited from the speed and reliability of real-time applications (Randegger and Hachler, 2001; Woodford *et al.*, 2002; Tan *et al.*, 2001; Hein *et al.*, 2001a; Shrestha *et al.*, 2002; Lindler *et al.*, 2001; Reischl *et al.*, 2000; Martineau *et al.*, 2000; Matsumura *et al.*, 2001; Gibson *et al.*, 1999; Chisholm *et al.*, 2001). These technologies have proven useful for determining the efficacy of antibiotic therapies when treating uncultivable organisms such as *Mycoplasma haemofelis* which infects cats (Tasker *et al.*, 2003, 2004). Meanwhile, the understanding and treatment of fulminant diseases such as meningitis, sepsis, inflammatory bowel disease and the sourcing of food poisoning outbreaks caused by characterised bacteria such as the group B *Streptococci*, *Mycobacterium* sp., *Escherichia coli*, *Bacteroides vulgatus* and *Salmonella* species have been enhanced by the speedy return of results from real-time and end-point fluorogenic assays (Taylor *et al.*, 2001; Bergeron *et al.*, 2000; Fujita *et al.*, 2002; Fortin *et al.*, 2001; Ibekwe *et al.*, 2002; Bellin *et al.*, 2001; Ke *et al.*, 2000; Chen *et al.*, 1997).

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* (Rauter *et al.*, 2002; Pietilä *et al.*, 2000; Pahl *et al.*, 1999), the methanotropic bio-remediating *Methylocystis* species (Kikuchi *et al.*, 2002) and bacteria capable of degrading agricultural herbicides such as the *Pseudomonas* sp. ADP and *Chelotobacter heintzii* (Devers *et al.*, 2004). The involvement of treponemes in the formation of periodontal disease has been studied using TaqMan chemistry, revealing a microbial role at every stage of disease (Asai *et al.*, 2002). In addition, measurement of the bacterial load of *Tropheryma whipplei* has permitted the discrimination of environmental contamination and low-level colonisation from active infection (Fenollar *et al.*, 2002).

Fungi, Parasites and Protozoans

A smaller but rapidly increasingly number of published applications have examined fungal, parasitic and protozoan pathogens of humans and plants. Real-time PCR assays have significantly contributed to the general diagnosis of invasive diseases, which are continually increasing with a rise in the population of immunocompromised patients. These pathogens include *Aspergillus fumigatus*, *Aspergillus flavus*, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida kefyr* and *Candida glabrata* (Brandt *et al.*, 1998; O'Sullivan *et al.*, 2003; Guiver and Oppenheim, 2001; Costa *et al.*, 2002). Monitoring the transcriptional activity of certain *Aspergillus nidulans* transporter genes has provided important information about their role in multidrug resistance (Semighini *et al.*, 2002). The homogeneous nature of real-time PCR has proven useful when assays have been employed to investigate buildings for the presence of potentially harmful levels of toxigenic fungal spores, or conidia, such as those produced by *Stachybotrys chartarum* (Roe *et al.*, 2001; Haugland *et al.*, 1999; Cruz-Perez *et al.*, 2001). Quantifying *Septoria tritici*, *Stagonospora nodorum*, *Puccinia striifomis* and *Puccinia recondita*, which cause blotch and rust in wheat crops, permitted crops at risk of full-blown disease to be identified so that specific fungicide treatments could be quickly employed (Fraaije *et al.*, 2001). Similar benefits have resulted from the detection of *Glomus mosseae*, *Phytophthora infestans* and *Phytophthora citricola* which infect and damage a wide range of plants including those used as food crops (Böhm *et al.*, 1999). Rapid molecular assays are playing an increased role in the detection and monitoring of fungicide resistance, the presence of compatible mating types, pathogenic variation within fungal populations and for airborne monitoring of dispersed pathogens (Zhang *et al.*, 1996; McCartney *et al.*, 2003).

Cryptosporidium parvum oocysts and the spores from *Encephalitozoon* species have been successfully genotyped or speciated using real-time PCR significantly improving upon laboratory diagnosis using microscopy and histochemical staining, especially for low concentrations of excreted material (Tanriverdi *et al.*, 2002; Wolk *et al.*, 2002).

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 detection of this microbe (Kupferschmidt *et al.*, 2001; Costa *et al.*, 2001). This technology is also useful to study how *Toxoplasma gondii* responds to antimicrobial therapies (Costa *et al.*, 2000).

Detection of malarial parasites using a mouse model in combination with real-time PCR has improved result turnaround and meant that parasite load data can be obtained (Bruña-Romero *et al.*, 2001; Witney *et al.*, 2001). Direct *in vivo* detection and quantitation of malarial parasites with a high level of sensitivity is also possible (Hermsen *et al.*, 2001; Lee *et al.*, 2002a) in addition to the indirect monitoring of stage-specific *Plasmodium falciparum* maturation by tracking the transcription of specific genes (Blair *et al.*, 2002).

Trichomonas vaginalis is the most common non-viral sexually transmitted organism in the world and the cost and time savings of real-time PCR assay have meant improvements to large-scale screening of patients at risk (Hardick *et al.*, 2003).

◆◆◆◆◆◆ DETECTING AGENTS OF BIOWARFARE

Perhaps no area of microbiology has impacted upon the community's psyche as much as the potential for microbes to be used as weapons of terror. While reported incidents of bioterrorism have to date been rare, further occurrences are considered by some to be inevitable and the potential for widespread disruption to communities is undoubtedly a serious threat to the population at large (Broussard, 2001). These disruptions need not take the form of widespread mortality, as small foci of morbidity would be sufficient to cause panic and disruption to essential services and financial markets. Hence, timely detection systems are an essential defence to minimise the effects of any such attack. Recently, there has been an explosion of literature indicating that real-time PCR is the tool of choice for rapidly detecting microbes used as agents of biological warfare. Many of these agents were, until recently, rarely encountered in the clinical laboratory and when they were, their detection relied upon relatively laborious and slow diagnostic techniques (Nulens and Voss, 2002). Nonetheless, this most recent area of application for real-time PCR technology enforces the role of real-time PCR as a new tool to complement the diagnostic arsenal rather than a complete replacement for traditional microbiological techniques.

There are three forms of human anthrax caused by *Bacillus anthracis*. These are the cutaneous, gastrointestinal and pulmonary forms. In contrast to self-limiting cutaneous anthrax, the ingestion or inhalation of endospores is generally fatal unless rapidly treated with antibiotics. The need for fast and reliable diagnostic tools became evident following the anthrax postal attacks late in 2001 in the United States. Real-time PCR assays permitted the rapid discrimination of weaponised pathogens from harmless laboratory-adapted or vaccine-related strains detecting *B. anthracis* spores and important plasmid or chromosomal markers (Makino *et al.*, 2001; Drago *et al.*, 2002; Uhl *et al.*, 2002; Oggioni *et al.*, 2002; Lee *et al.*, 1999b; Qi *et al.*, 2001). These assays could also discriminate pathogenic from mildly or apathogenic *Bacillus* spp. taken from colonies or enrichment broths (Ellerbrok *et al.*, 2002; Hurtle *et al.*, 2004). The addition of hand-held miniaturised real-time instruments and mobile laboratory systems has further enhanced the detection of bioweapons by providing the potential for on-site results in under 30 min during emergency situations (Higgins *et al.*, 2003a,b). Additionally *Francisella tularensis*, the cause of tularemia and *Yersinia pestis* the causative agent of plague have been detected using nuclease or HybProbe assays (Lindler *et al.*, 2001; Lindler and Fan, 2003). In some instances these assays can be modified for use in the field, permitting rapid and highly specific detection at the point of concern (Higgins *et al.*, 1998, 2000).

Smallpox is caused by infection with variola virus (VARV), a member of the family *Poxviridae*, genus *Orthopoxvirus*. VARV evokes the most serious concerns as a biowarfare agent because of low immunity among the population and the application of real-time PCR is attempting to address the diagnostic issues (Ibrahim *et al.*, 2003). The disease was declared eradicated in 1979 by a global vaccination program with the last naturally occurring VARV infection occurring in 1977 in Somalia (WHO, 1980; Behbehani, 1983). While humans are the only natural host for VARV, both vaccinia virus and cowpox virus have a much broader host spectrum. Although zoonotic infections of humans by monkeypox and other members of the genus *Orthopoxvirus* occur, potential VARV infections bear by far the most lethal risk for man (Centers for Disease Control and Prevention, 2003; Czerny *et al.*, 1991; Lewis-Jones, 2002; Stephenson, 2003). Due to this infectious potential, VARV has recently been flagged as a biological weapons threat (Henderson *et al.*, 1999; Whitley, 2003). The highest degree of sequence homology among characterised members of the genus *Orthopoxvirus* is found towards the centre of the genome while the terminal regions can exhibit considerable variability. Clearly, rapid and sensitive identification of variola virus and its discrimination from other members of the genus is fundamental to reliable diagnosis and risk evaluation of environmental samples. However, the consequences of a false positive or a false negative PCR result in smallpox diagnosis caused by unexpected sequence variation is considerable; therefore, identification of variola virus should also benefit from amplification of several independent targets by PCR.

Conventional VARV typing and sequencing assays may take more than 48 h to complete and, for obvious reasons, this is an unacceptable lag period. Even the use of end-point fluorescence detection decreases assay times drastically (Ibrahim *et al.*, 1997). Variola virus has also been used as a target to test new field-portable real-time PCR instruments in combination with rapid nucleic acid extraction techniques demonstrating their potential for detecting potential bioweapons on site (Ibrahim *et al.*, 1998). However, these applications have once again highlighted the importance of careful assay design when discriminating highly pathogenic microbes from innocuous, but closely related species (Espy *et al.*, 2002; Nitsche *et al.*, 2004, in press). This is an issue that clearly highlights technical difficulties, which remain in the area of diagnostic PCR design. In many cases gene sequencing is still the final stage for confirming a provisional identification of microbial material from a site or patient suspected of infection with a bioweapon.

◆◆◆◆◆◆ HOST IMMUNITY: MEASURING THE RESPONSE TO A MICROBE

The application of real-time PCR is beginning to provide evidence to support paradigm shifts in the way we conceptualise the interplay between microbe and host and in the way we describe these interactions

(Casadevall and Pirofski, 2003). Pathogen-centric views have dogmatically defined virulence as the result of microbial factors, when in fact the role of the host can be clearly shown to exert a significant influence (Casadevall and Pirofski, 2001; Mitchell, 1998). Increasingly, the old view is being challenged by our growing understanding that changing host immunity is as integral to the course of an infectious disease as the microbe causing the infection. This poses the question "what is a pathogen"? (Casadevall and Pirofski, 2002). Real-time PCR is helping formulate answers to this and other questions by defining the fitness of the host though quantitation of the immune response to infection and antimicrobial therapies and also as a tool to quantify the damage caused. While micro-array technologies provide a comprehensive snap-shot of the state of the host or invading microbe's transcriptome, arrays do not permit reliable quantitation of the target change over a broad dynamic range (Lockhart and Winzeler, 2000; Clewley, 2004; Lucchini *et al.*, 2001). Real-time PCR has increasingly become the method of choice for validating and further characterising experimental data generated by micro-arrays and is the favoured tool for determining gene transcript abundance in basic research, molecular medicine and biotechnology. These determinations are essential markers of microbial–host interaction. While Northern blotting and RNase protection can determine both the size and amount of a transcript, the former is relatively insensitive, and both require large amounts of starting template (Wang and Brown, 1999). Considerably more has been published on the role of real-time PCR in the basic detection of cytokine transcripts from stored or *in vitro* stimulated blood cells than as an indicator of microbial activation (Kruse *et al.*, 1997; Härtel *et al.*, 1999; Blaschke *et al.*, 2000; Stordeur *et al.*, 2002). This is set to be a growth area for future real-time PCR applications.

Cytokines constitute the majority of mediators involved in the innate mammalian immune response recognising microbes, communicating with and recruiting leukocytes and removing invading microorganisms. However, it is the host's molecular pattern recognition systems operating via specialised receptors which trigger many of the events leading to cytokine induction post-infection (Strieter *et al.*, 2003). Real-time PCR has identified critical mRNA from these receptors and from cytokine genes as a *de facto* indicator of protein production (Overbergh *et al.*, 1999, 2003; Giulietti *et al.*, 2001; Hein *et al.*, 2001b). While this is not a perfect relationship insofar as protein levels are modified by more than simple transcript availability, studies have shown a good correlation between the transcriptome and the proteome for many genes (Balnaves *et al.*, 1995). Additionally, it is often impossible to detect the expressed product in tissue samples due to the low expression levels and short half-lives of cytokine proteins (Broberg *et al.*, 2003; Stordeur *et al.*, 2002). Nonetheless, transcriptional studies using real-time PCR are enhanced by protein expression data.

Well-controlled quantitative real-time PCR experiments have permitted the differentiation and quantitation of IFN-α subclasses as a measure of the innate immune response to infectious or inactivated virus in humans (Löseke *et al.*, 2003). The expression of other cytokines has also

been monitored in murine models of microbial immunopathogenesis (Deng *et al.*, 2003; Broberg *et al.*, 2003). Microbial load studies in concert with cytokine protein detection have demonstrated relationships that may play a role in predicting the course and severity of post-transplant lymphoproliferative disease in EBV-positive patients (Muti *et al.*, 2003). The direct impact of cytokines on viral gene expression and the effect of viral cytokine homologues on co-infecting viruses have potentially established new therapeutic strategies for diseases (Song *et al.*, 2002).

Rapid advances in the performance and variety of fluorogenic chemistries and real-time PCR platforms together with the exponential increase in our understanding of the process have ensured real-time PCR is as important a technology for the diagnostic and research microbiology laboratory of tomorrow as agarose gel electrophoresis was to the laboratory of yesterday. Real-time PCR is no more or less than a diagnostic tool and therefore the data it generates are only as reliable as the design and implementation of each assay. Judicious application of the technology will both simplify and hasten the search for answers to many complex experimental and diagnostic questions. In order to more accurately understand the process of virulence, such reliable and robust tools will be essential for defining the interplay between the host and the invading microbe, and when necessary, to do so in a clinically relevant period of time.

References

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiba, M. and Kohara, M. (1999). Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J. Clin. Microbiol.* **37**, 2899–2903.

Aberham, C., Pendl, C., Gross, P., Zerlauth, G. and Gessner, M. (2001). A quantitative, internally controlled real-time PCR assay for the detection of parvovirus B19 DNA. *J. Virol. Meth.* **92**, 183–191.

Afonina, I. A., Reed, M. W., Lusby, E., Shishkina, I. G. and Belousov, Y. S. (2002a). Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. *Biotechniques* **32**, 940–949.

Afonina, I. A., Sanders, S., Walburger, D. and Belousov, Y. S. (2002b). Accurate SNP typing by real-time PCR. *Pharmagenomics* Jan/Feb, 48–54.

Ahmed, F. E. (2002). Detection of genetically modified organisms in foods. *Trends Biotechnol.* **20**, 215–223.

Aldous, E. W., Collins, M. S., McGoldrick, A. and Alexander, D. J. (2001). Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay. *Vet. Microbiol.* **80**, 201–212.

Alexandersen, S., Oleksiewicz, M. B. and Donaldson, A. I. (2001). The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT-PCR. *J. Gen. Virol.* **82**, 747–755.

Alexandre, I., Zammatteo, N., Ernest, I., Ladriere, J.-M., Hamels, Le. S., Chandelier, N., Vipond, B. and Remacle, J. (1998). Quantitative determination of CMV DNA using a combination of competitive PCR amplification and sandwich hybridization. *Biotechniques* **25**, 676–683.

Applegate, T. L., Iland, H. J., Mokany, E. and Todd, A. V. (2002). Diagnosis and molecular monitoring of acute promyelocytic leukemia using DzyNA reverse transcription-PCR to quantify PML/RARα fusion transcripts. *Clin. Chem.* **48**, 1338–1343.

Argaw, T., Ritzhaupt, A. and Wilson, C. A. (2002). Development of a real time quantitative PCR assay for detection of porcine endogenous retrovirus. *J. Virol. Meth.* **106**, 97–106.

Asai, Y., Jinno, T., Igarashi, H., Ohyama, Y. and Ogawa, T. (2002). Detection and quantification of oral treponemes in subgingival plaque by real-time PCR. *J. Clin. Microbiol.* **40**, 3334–3340.

Bae, H. G., Nitsche, A., Teichmann, A., Biel, S. S. and Niedrig, M. (2003). Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. *J. Virol. Meth.* **110**, 185–191.

Bagnarelli, P., Menzo, S., Valenza, A., Paolucci, S., Petroni, S., Scalise, G., Sampaolesi, R., Manzin, A. and Clementi, M. (1995). Quantitative molecular monitoring of human immunodeficiency virus type 1 activity during therapy with specific antiretroviral compounds. *J. Clin. Microbiol.* **33**, 16–23.

Ballard, A. L., Fry, N. K., Chan, L., Surman, S. B., Lee, J. V., Harrison, T. G. and Towner, K. J. (2000). Detection of *Legionella pneumophila* using real-time PCR hybridisation assay. *J. Clin. Microbiol.* **38**, 4215–4218.

Balnaves, M. E., Bonacquisto, L., Francis, I., Glazner, J. and Forrest, S. (1995). The impact of newborn screening on cystic fibrosis testing in Victoria. *Austr. J. Med. Genet.* **32**, 537–542.

Barany, F. (1991). The ligase chain reaction in a PCR world. *PCR Meth. Appl.* **1**, 5–16.

Barratt, K. and Mackay, J. F. (2002). Improving real-time PCR genotyping assays by asymmetric amplification. *J. Clin. Microbiol.* **40**, 1571–1572.

Barzon, L., Bonaguro, R., Castagliuolo, I., Chilosi, M., Franchin, E., Del Vecchio, C., Giaretta, I., Boscaro, M. and Palù, G. (2003). Gene therapy of thyroid cancer via retrovirally-driven combined expression of human interleukin-2 and herpes simplex virus thymidine kinase. *Eur. J. Endocrinol.* **148**, 73–80.

Bassler, H. A., Flood, S. J. A., Livak, K. J., Marmaro, J., Knorr, R. and Batt, C. A. (1995). Use of a fluorogenic probe in a PCR-based assay for the detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **61**, 3724–3728.

Beames, B., Chavez, D., Guerra, B., Notvall, L., Brasky, K. M. and Lanford, R. E. (2000). Development of a primary tamarin hepatocyte culture system for GB virus-B: a surrogate model for hepatitis C virus. *J. Virol.* **74**, 11764–11772.

Becker-Andre, M. and Hahlbrock, K. (1989). Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res.* **17**, 9437–9447.

Behbehani, A. M. (1983). The smallpox story: life and death of an old disease. *Microbiol. Rev.* **47**, 455–509.

Bellin, T., Pulz, M., Matussek, A., Hempen, H.-G. and Gunzer, F. (2001). Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. *J. Clin. Microbiol.* **39**, 370–374.

Bengtsson, M., Karlsson, H. J., Westman, G. and Kubista, M. (2003). A new minor groove binding asymmetric cyanine reporter dye for real-time PCR. *Nucleic Acids Res.* **31**, e45.

Bergeron, M. G., Ke, D., Ménard, C., Picard, F. J., Gagnon, M., Bernier, M., Ouellette, M., Roy, P. H., Marcoux, S. and Fraser, W. D. (2000). Rapid detection of group B streptococci in pregnant women at delivery. *N. Engl. J. Med.* **343**, 175–179.

Bernard, P. S. and Wittwer, C. T. (2000). Homogeneous amplification and variant detection by fluorescent hybridization probes. *Clin. Chem.* **46**, 147–148.

Bernard, P. S., Lay, M. J. and Wittwer, C. T. (1998). Integrated amplification and detection of the C677T point mutation in the methyltetrahydrofolate reductase gene by fluorescence resonance energy transfer and probe melting curves. *Anal. Biochem.* **255**, 101–107.

Biel, S. S. and Madeley, D. (2001). Diagnostic virology – the need for electron microscopy: a discussion paper. *J. Clin. Virol.* **22**, 1–9.

Biel, S. S., Held, T. K., Landt, O., Niedrig, M., Gelderblom, H. R., Siegert, W. and Nitsche, A. (2000). Rapid quantification and differentiation of human polyomavirus DNA in undiluted urine from patients after bone marrow transplantation. *J. Clin. Microbiol.* **38**, 3689–3695.

Biggar, R. J., Whitby, D., Marshall, V., Linhares, A. C. and Black, F. (2000). Human herpesvirus 8 in Brazilian Amerindians: a hyperendemic population with a new subtype. *J. Infect. Dis.* **181**, 1562–1568.

Blair, P. L., Witney, A., Haynes, J. D., Moch, J. K., Carucci, D. J. and Adams, J. H. (2002). Transcripts of developmentally regulated *Plasmodium falciparum* genes quantified by real-time RT-PCR. *Nucleic Acids Res.* **30**, 2224–2231.

Blaschke, V., Reich, K., Blaschke, S., Zipprich, S. and Neumann, C. (2000). Rapid quantitation of proinflammatory and chemoattractant cytokine expression in small tissue samples and monocyte-derived dendritic cells: validation of a new real-time RT-PCR technology. *J. Immunol. Meth.* **246**, 79–90.

Boivin, G., Côte, S., Déry, P., De Serres, G. and Bergeron, M. G. (2004). Multiplex real-time PCR assay for detection of influenza and human respiratory syncytial virus. *J. Clin. Microbiol.* **42**, 45–51.

Böhm, J., Hahn, A., Schubert, R., Bahnweg, G., Adler, N., Nechwatal, J., Oehlmann, R. and Oswald, W. (1999). Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytophthora citricola* in their respective host plants. *J. Phytopathol.* **147**, 409–416.

Borson, N. D., Strausbauch, M. A., Wettstein, P. J., Oda, R. P., Johnston, S. L. and Landers, J. P. (1998). Direct quantitation of RNA transcripts by competitive single-tube RT-PCR and capillary electrophoresis. *Biotechniques* **25**, 130–137.

Brandt, M. E., Padhye, A. A., Mayer, L. W. and Holloway, B. P. (1998). Utility of random amplified polymorphic DNA PCR and TaqMan automated detection in molecular identification of *Aspergillus fumigatus*. *J. Clin. Microbiol.* **36**, 2057–2062.

Brechtbuehl, K., Whalley, S. A., Dusheiko, G. M. and Saunders, N. A. (2001). A rapid real-time quantitative polymerase chain reaction for hepatitis B virus. *J. Virol. Meth.* **93**, 105–113.

Bretagne, S. (2003). Molecular diagnostics in clinical parasitology and mycology: limits of the current polymerase chain reaction (PCR) assays and interest of the real-time PCR assays. *Clin. Microbiol. Infect.* **9**, 505–511.

Brightwell, G., Pearce, M. and Leslie, D. (1998). Development of internal controls for PCR detection of *Bacillus anthracis*. *Mol. Cell. Probes* **12**, 367–377.

Broberg, E. K., Nygårdas, M., Salmi, A. A. and Hukkanen, V. (2003). Low copy number detection of herpes simplex virus type 1 mRNA and mouse Th1 type cytokine mRNAs by Light Cycler quantitative real-time PCR. *J. Virol. Meth.* **112**, 53–65.

Brorson, K., Swann, P. G., Lizzio, E., Maudru, T., Peden, K. and Stein, K. E. (2001). Use of a quantitative product-enhanced reverse transcriptase assay to monitor retrovirus levels in mAb cell-culture and downstream processing. *Biotechnol. Prog.* **17**, 188–196.

Broussard, L. A. (2001). Biological agents: weapons of warfare and bioterrorism. *Mol. Diagn.* **6**, 323–333.

Bruña-Romero, O., Hafalla, J. C. R., González-Aseguinolaza, G., Sano, G., Tsuji, M. and Zavala, F. (2001). Detection of malaria liver-stages in mice infected through the bite of a single *Anopheles* mosquito using a highly sensitive real-time PCR. *Int. J. Parasitol.* **31**, 1499–1502.

Burgart, L. J., Robinson, R. A., Heller, M. J., Wilke, W. W., Iakoubova, O. K. and Cheville, J. C. (1992). Multiplex polymerase chain reaction. *Mod. Pathol.* **5**, 320–323.

Burgos, J. S., Ramírez, C., Tenorio, R., Sastre, I. and Bullido, M. J. (2002). Influence of reagents formulation on real-time PCR parameters. *Mol. Cell. Probes* **16**, 257–260.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**, 169–193.

Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29**, 23–39.

Callahan, J. D., Wu, S.-J. L., Dion-Schultz, A., Mangold, B. E., Peruski, L. F., Watts, D. M., Porter, K. R., Murphy, G. R., Suharyono, W., King, C.-C., Hayes, C. G. and Temenak, J. J. (2001). Development ad evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *J. Clin. Microbiol.* **39**, 4119–4124.

Cane, P. A., Cook, P., Ratcliffe, D., Mutimer, D. and Pillay, D. (1999). Use of real-time PCR and fluorimetry to detect lamivudine resistance-associated mutations in hepatitis B virus. *Antimicrob. Agent Chem.* **43**, 1600–1608.

Capone, R. B., Pai, S. I., Koch, W. M. and Gillison, M. L. (2001). Detection and quantitation of human papillomavirus (HPV) DNA in the sera of patients with HPV-associated head and neck squamous cell carcinoma. *Clin. Cancer Res.* **6**, 4171–4175.

Cardullo, R. A., Agrawai, S., Flores, C., Zamecnik, P. C. and Wolf, D. E. (1988). Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. *Proc. Natl Acad. Sci. USA* **85**, 8790–8794.

Carman, B. (2001). Molecular techniques should now replace cell culture in diagnostic virology laboratories. *Rev. Med. Virol.* **11**, 347–349.

Carman, W. F., Wallace, L. A., Walker, J., McIntyre, S., Noone, A., Christie, P., Millar, J. and Douglas, J. D. (2000). Rapid virological surveillance of community influenza infection in general practice. *Br. Med. J.* **321**, 736–737.

Casadevall, A. and Pirofski, L. (2001). Host pathogen interactions: the attributes of virulence. *J. Infect. Dis.* **184**, 337–344.

Casadevall, A. and Pirofski, L. (2002). What is a pathogen? *Ann. Med.* **34**, 2–4.

Casadevall, A. and Pirofski, L. (2003). Microbial virulence results from the interaction between host and microorganism. *Trends Microbiol.* **11**, 157–158.

Celi, F. S., Mentuccia, D., Proietti-Pannunzi, L., di Gioia, C. R. T. and Andreoli, M. (2000). Preparing poly-A-containing RNA internal standards for multiplex competitive RT-PCR. *Biotechniques* **29**, 454–458.

Centers for Disease Control and Prevention, (2003). Multistate outbreak of monkeypox – Illinois, Indiana and Wisconsin. *MMWR* **52**, 537–540.

Chamberlain, J. S., Gibbs, R. A., Ranier, J. E., Nguyen, P. N. and Caskey, C. T. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res.* **16**, 11141–11156.

Chandelier, A., Dubois, N., Baelen, F., De Leener, F., Warnon, S., Remacle, J. and Lepoivre, P. (2001). RT-PCR-ELOSA tests on pooled sample units for the detection of virus Y in potato tubers. *J. Virol. Meth.* **91**, 99–108.

Chang, L.-J., Urlacher, V., Iwakuma, T., Cui, Y. and Zucali, J. (1999). Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther.* **6**, 715–728.

Chehab, F. F., Doherty, M., Cai, S., Kan, Y. W., Cooper, S. and Rubin, E. M. (1987). Detection of sickle cell anaemia and thalassaemias. *Nature* **329**, 293–294.

Chen, S., Yee, A., Griffiths, M., Larkin, C., Yamashiro, C. T., Behari, R., Paszko-Kolva, C., Rahn, K. and De Grandis, S. A. (1997). The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. *Int. J. Food Microbiol.* **35**, 239–250.

Chen, R. W., Piiparinen, H., Seppänen, M., Koskela, P., Sarna, S. and Lappalainen, M. (2001). Real-time PCR for detection and quantitation of hepatitis B virus DNA. *J. Med. Virol.* **65**, 250–256.

Chen, Y. P., Higgins, J. A. and Gundersen-Rindal, D. E. (2003). Quantitation of a *Glyptapanteles indiensis* polydnavirus gene expressed in parasitized host, *Lymantria dispar*, by real-time quantitative RT-PCR. *J. Virol. Meth.* **114**, 125–133.

Chisholm, S. A., Owen, R. J., Teare, E. L. and Saverymuttu, S. (2001). PCR-based diagnosis of *Helicobacter pylori* infection and real-time determination of clarithromycin resistance directly from human gastric biopsy samples. *J. Clin. Microbiol.* **39**, 1217–1220.

Choo, C. K., Ling, M. T., Suen, C. K. M., Chan, K. W. and Kwong, Y. L. (2000). Retrovirus-mediated delivery of HPV16 E7 antisense RNA inhibited tumorigenicity of CaSki cells. *Gynecol. Oncol.* **78**, 293–301.

Chou, Q., Russell, M., Birch, D. E., Raymond, J. and Bloch, W. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res.* **20**, 1717–1723.

Clarke, S. C., Reid, J., Thom, L., Denham, B. C. and Edwards, G. F. S. (2002). Laboratory confirmation of meningococcal disease in Scotland, 1993–9. *J. Clin. Pathol.* **55**, 32–36.

Clegg, R. M. (1992). Fluorescence resonance energy transfer and nucleic acids. *Meth. Enzymol.* **211**, 353–388.

Clementi, M. (2000). Quantitative molecular analysis of virus expression and replication. *J. Clin. Microbiol.* **38**, 2030–2036.

Clementi, M., Menzo, S., Manzin, A. and Bagnarelli, P. (1995). Quantitative molecular methods in virology. *Arch. Virol.* **140**, 1523–1539.

Clewley, J. P. (2004). A role for arrays in clinical virology: fact or fiction? *J. Clin. Virol.* **29**, 2–12.

Cockerill, F. R. III and Smith, T. F. (2002). Rapid-cycle real-time PCR: a revolution for clinical microbiology. *ASM News* **68**, 77–83.

Compton, J. (1991). Nucleic acid sequence-based amplification. *Nature* **350**, 91–92.

Corbella, M. E. and Puyet, A. (2003). Real-time reverse transcription-PCR analysis of expression of halobenzoate and salicylate catabolism-associated operons in two strains of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **69**, 2269–2275.

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J. and Kaczmarski, E. B. (2001). Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J. Clin. Microbiol.* **39**, 1553–1558.

Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J., Kaczmarski, E. B. and Mutton, K. J. (2002). Development and evaluation of a "real-time" RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. *J. Med. Virol.* **67**, 555–562.

Costa, J.-M., Pautas, C., Ernault, P., Foulet, F., Cordonnier, C. and Bretagne, S. (2000). Real-time PCR for diagnosis and follow-up of toxoplasma reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. *J. Clin. Microbiol.* **38**, 2929–2932.

Costa, J.-M., Ernault, P., Gautier, E. and Bretagne, S. (2001). Prenatal diagnosis of congenital toxoplasmosis by duplex real-time PCR using fluorescence resonance energy transfer hybridization probes. *Prenat. Diagn.* **21**, 85–88.

Costa, C., Costa, J.-M., Desterke, C., Botterel, F., Cordonnier, C. and Bretagne, S. (2002). Real-time PCR coupled with automated DNA extraction and detection of galactomannan antigen in serum by enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis. *J. Clin. Microbiol.* **40**, 2224–2227.

Côte, S., Abed, Y. and Boivin, G. (2003). Comparative evaluation of real-time PCR assays for detection of the human metapneumovirus. *J. Clin. Microbiol.* **41**, 3631–3635.

Creelan, J. L. and McCullough, S. J. (2000). Evaluation of strain-specific primer sequences from an abortifacient strain of ovine *Chlamydophila abortus* (*Chlamydia psittaci*) for the detection of EAE by PCR. *FEMS Microbiol. Lett.* **190**, 103–108.

Crockett, A. O. and Wittwer, C. T. (2001). Fluorescein-labelled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides. *Anal. Biochem.* **290**, 89–97.

Cruz-Perez, P., Buttner, M. P. and Stetzenbach, L. D. (2001). Specific detection of *Stachybotrys chartarum* in pure culture using quantitative polymerase chain reaction. *Mol. Cell. Probes* **15**, 129–138.

Czerny, C. P., Eis-Hubinger, A. M., Mayr, A., Schneweis, K. E. and Pfeiff, B. (1991). Animal poxviruses transmitted from cat to man: current event with lethal end. *Zentralbl. Veterinarmed. B* **38**, 421–431.

Daniels, P., Ksiazek, T. and Eaton, B. T. (2001). Laboratory diagnosis of Nipah and Hendra virus infections. *Microbes Infect.* **3**, 289–295.

de Viedma, D. G., Diaz Infanntes, M., Lasala, F., Chaves, F., Alcalá, L. and Bouza, E. (2002). New real-time PCR able to detect in a single tube multiple rifamin resistance mutations and high-level isoniazid resistance mutations in *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **40**, 988–995.

de Wit, C., Fautz, C. and Xu, Y. (2000). Real-time quantitative PCR for retrovirus-like particle formation in CHO cell culture. *Biologicals* **28**, 137–148.

DeGraves, F. J., Gao, D. and Kaltenboeck, B. (2003). High-sensitivity quantitative PCR platform. *Biotechniques* **34**, 106–115.

Dekonenko, A., Ibrahim, M. S. and Schmaljohn, C. S. (1997). A colorimetric PCR–enzyme immunoassay to identify hantaviruses. *Clin. Diag. Virol.* **8**, 113–121.

Deng, X., Li, H. and Tang, Y.-W. (2003). Cytokine expression in respiratory syncytial virus-infected mice as measured by quantitative reverse-transcriptase PCR. *J. Virol. Meth.* **107**, 141–146.

Desjardin, L. E., Chen, Y., Perkins, M. D., Teixeira, L., Cave, M. D. and Eisenach, K. D. (1998). Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis. *J. Clin. Microbiol.* **36**, 1964–1968.

Devers, M., Soulas, G. and Martin-Laurent, F. (2004). Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. *J. Microbiol. Meth.* **56**, 3–15.

Didenko, V. V. (2001). DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. *Biotechniques* **31**, 1106–1121.

Drago, L., Lombardi, A., De Vecchi, E. and Gismondo, M. R. (2002). Real-time PCR assay for the rapid detection of *Bacillus anthracis* spores in clinical samples. *J. Clin. Microbiol.* **40**, 4399.

Drosten, C., Gunther, S., Preiser, W., van der, W. S., Brodt, H. R., Becker, S., Rabenau, H., Panning, M., Kolesnikova, L., Fouchier, R. A., Berger, A., Burguiere, A. M., Cinatl, J., Eickmann, M., Escriou, N., Grywna, K., Kramme, S., Manuguerra, J. C., Muller, S., Rickerts, V., Sturmer, M., Vieth, S., Klenk, H. D., Osterhaus, A. D., Schmitz, H. and Doerr, H. W. (2003). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**, 1967–1976.

Echevarria, J. E., Erdman, D. D., Swierkosz, E. M., Holloway, B. P. and Anderson, L. J. (1998). Simultaneous detection and identification of human parainfluenza viruses 1, 2, and 3 from clinical samples by multiplex PCR. *J. Clin. Microbiol.* **36**, 1388–1391.

Edelman, D. C. and Barletta, J. (2003). Real-time PCR provides improved detection and titer determination of bacteriophage. *Biotechniques* **35**, 368–375.

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B. and Nielsen, P. E. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen bonding rules. *Nature* **365**, 566–568.

Eishi, Y., Suga, M., Ishige, I., Kobayashi, D., Yamada, T., Takemura, T., Takizawa, T., Koike, M., Kudoh, S., Costabel, U., Guzman, J., Rizzato, G., Gambacorta, M., du Bois, R., Nicholson, A. G., Sharma, O. P. and Ando, M. (2002). Quantitative analysis of mycobacterial and propionibacterial DNA in lymph nodes of Japanese and European patients with sarcoidosis. *J. Clin. Microbiol.* **40**, 198–204.

Elenitoba, K. S. J., Bohling, S. D., Wittwer, C. T. and King, T. C. (2001). Multiplex PCR by multicolor fluorimetry and fluorescence melting curve analysis. *Nat. Med.* **7**, 249–253.

Ellerbrok, H., Nattermann, H., Özel, M., Beutin, L., Appel, B. and Pauli, G. (2002). Rapid and sensitive identification of pathogenic and apathogenic *Bacillus anthracis* by real-time PCR. *FEMS Microbiol. Lett.* **214**, 51–59.

Ellis, J. S. and Zambon, M. C. (2002). Molecular diagnosis of influenza. *Rev. Med. Virol.* **12**, 375–389.

Emery, S. L., Erdman, D. D., Bowen, M. D., Newton, B. R., Winchell, J. M., Meyer, R. F., Tong, S., Cook, B. T., Holloway, B. P., McCaustland, K. A., Rota, P. A., Bankamp, B., Lowe, L. E., Ksiazek, T. G., Bellini, W. J. and Anderson, L. J. (2004). Real-time reverse transcription-polymerase chain reaction assay for SARS-associated Coronavirus. *Emerg. Infect. Dis.* **10**, 311–316.

Espy, M. J., Ross, T. K., Teo, R., Svien, K. A., Wold, A. D., Uhl, J. R. and Smith, T. F. (2000a). Evaluation of LightCycler PCR for implementation of laboratory diagnosis of herpes simplex virus infections. *J. Clin. Microbiol.* **38**, 3116–3118.

Espy, M. J., Uhl, J. R., Mitchell, P. S., Thorvilson, J. N., Svien, K. A., Wold, A. D. and Smith, T. F. (2000b). Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR. *J. Clin. Microbiol.* **38**, 795–799.

Espy, M. J., Cockerill, F. R. III, Meyer, R. F., Bowen, M. D., Poland, G. A., Hadfield, T. L. and Smith, T. F. (2002). Detection of smallpox virus DNA by LightCycler PCR. *J. Clin. Microbiol.* **40**, 1985–1988.

Fan, H. and Gulley, M. L. (2001). Epstein-Barr viral load measurement as a marker of EBV-related disease. *Mol. Diagn.* **6**, 279–289.

Fenollar, F., Fournier, P.-E., Raoult, D., Gérolami, R., Lepidi, H. and Poyart, C. (2002). Quantitative detection of *Tropheryma whipplei* DNA by real-time PCR. *J. Clin. Microbiol.* **40**, 1119–1120.

Fernandez, C., Boutolleau, D., Manichanh, C., Mangeney, N., Agut, H. and Gautheret-Dejean, A. (2002). Quantitation of HHV-7 genome by real-time polymerase chain reaction assay using MGB probe technology. *J. Virol. Meth.* **106**, 11–16.

Fiandaca, M. J., Hyldig-Nielsen, J. J., Gildea, B. D. and Coull, J. M. (2001). Self-reporting PNA/DNA primers for PCR analysis. *Genome Res.* **11**, 609–611.

Förster, T. (1948). Zwischenmolekulare energiewanderung und fluoreszenz. *Ann. Phys.* **6**, 55–75.

Fortin, N. Y., Mulchandani, A. and Chen, W. (2001). Use of real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* 0157:H7. *Anal. Biochem.* **289**, 281–288.

Fraaije, B. A., Lovell, D. J., Coelho, J. M., Baldwin, S. and Hollomon, D. W. (2001). PCR-based assays to assess wheat varietal resistance to blotch (*Septoria tritici* and *Stagonospora nodorum*) and rust (*Puccinia striiformis* and *Puccinia recondita*) diseases. *Eur. J. Plant Pathol.* **107**, 905–917.

Fredricks, D. N. and Relman, D. A. (1999). Application of polymerase chain reaction to the diagnosis of infectious disease. *Clin. Infect. Dis.* **29**, 475–488.

Freeman, W. M., Walker, S. J. and Vrana, K. E. (1999). Quantitative RT-PCR: pitfalls and potential. *Biotechniques* **26**, 112–125.

French, D. J., Archard, C. L., Brown, T. and McDowell, D. G. (2001). HyBeacon™ probes: a new tool for DNA sequence detection and allele discrimination. *Mol. Cell. Probes* **15**, 363–374.

French, D. J., Archard, C. L., Andersen, M. T. and McDowell, D. G. (2002). Ultra-rapid analysis using HyBeacon™ probes and direct PCR amplification from saliva. *Mol. Cell. Probes* **16**, 319–326.

Freymuth, F., Eugene, G., Vabret, A., Petitjean, J., Gennetay, E., Brouard, J., Duhamel, J. F. and Guillois, B. (1995). Detection of respiratory syncytial virus by reverse transcription-PCR and hybridization with a DNA enzyme immunoassay. *J. Clin. Microbiol.* **33**, 3352–3355.

Fujita, H., Eishi, Y., Ishige, I., Saitoh, K. and Takizawa, T. (2002). Quantitative analysis of bacterial DNA from *Mycobacteria* spp. *Bacteroides vulgatus*, and *Escherichia coli* in tissue samples from patients with inflammatory bowel diseases. *J. Gastroenterol.* **37**, 509–516.

Furuta, Y., Ohtani, F., Sawa, H., Fukuda, S. and Inuyama, Y. (2001). Quantitation of varicella-zoster virus DNA in patients with Ramsay Hunt syndrome and zoster sine herpete. *J. Clin. Microbiol.* **39**, 2856–2859.

Gallagher, A., Armstrong, A. A., MacKenzie, J., Shield, L., Khan, G., Lake, A., Proctor, S., Taylor, P., Clements, G. B. and Jarrett, R. F. (1999). Detection of epstein-barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. *Int. J. Can.* **84**, 442–448.

Gault, E., Michel, Y., Nicolas, J.-C., Belabani, C., Nicolas, J.-C. and Garbarg-Chenon, A. (2001). Quantification of human cytomegalovirus DNA by real-time PCR. *J. Clin. Microbiol.* **39**, 772–775.

Gelmini, S., Orlando, C., Sestini, R., Vona, G., Pinzani, P., Ruocco, L. and Pazzagli, M. (1997). Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure c-cerB-2 oncogene amplification. *Clin. Chem.* **43**, 752–758.

Gerard, C. J., Arboleda, M. J., Solar, G., Mule, J. J. and Kerr, W. G. (1996). A rapid and quantitative assay to estimate gene transfer into retrovirally transduced hematopoietic stem/progenitor cells using a 96-well format PCR and fluorescent detection system universal for MMLV-based proviruses. *Hum. Gen. Ther.* **7**, 343–354.

Gibb, T. R., Norwood, D. A. Jr., Woollen, N. and Henchal, E. A. (2001a). Development and evaluation of a fluorogenic 5′-nuclease assay to identify Marburg virus. *Mol. Cell. Probes* **15**, 259−266.

Gibb, T. R., Norwood, D. A. Jr., Woollen, N. and Henchal, E. A. (2001b). Development and evaluation of a fluorogenic 5′ nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. *J. Clin. Microbiol.* **39**, 4125−4130.

Gibson, U. E. M., Heid, C. A. and Williams, P. M. (1996). A novel method for real time quantitative RT-PCR. *Genome Res.* **6**, 995−1001.

Gibson, J. R., Saunders, N. A. and Owen, R. J. (1999). Novel method for rapid determination of clarithromycin sensitivity in *Helicobacter pylori*. *J. Clin. Microbiol.* **37**, 3746−3748.

Giglio, S., Monis, P. T. and Saint, C. P. (2003). Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. *Nucleic Acids Res.* **31**, e136.

Gilliland, G., Perrin, S. and Bunn, H. F. (1990). *Competitive PCR for Quantitation of mRNA. PCR Protocols: A Guide to Methods and Applications*. Academic Press, New York, pp. 60−69.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR: an emerging technology hits mainstream. *Exp. Hematol.* **30**, 503−512.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. and Mathieu, C. (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**, 386−401.

Glasel, J. A. (1995). Validity of nucleic acid purities monitored by 260 nm/280 nm absorbance ratios. *Biotechniques* **18**, 62−63.

Goerke, C., Bayer, M. G. and Wolz, C. (2001). Quantification of bacterial transcripts during infection using competitive reverse transcription-PCR (RT-PCR) and LightCycler RT-PCR. *Clin. Diag. Lab. Immunol.* **8**, 279−282.

Gruber, F., Falkner, F. G., Dorner, F. and Hämmerle, T. (2001). Quantitation of viral DNA by real-time PCR applying duplex amplification, internal standardization, and two-colour fluorescence detection. *Appl. Environ. Microbiol.* **67**, 2837−2839.

Guiver, M. and Oppenheim, B. A. (2001). Rapid identification of *Candida* species by TaqMan PCR. *J. Clin. Pathol.* **54**, 362−366.

Gundry, C. N., Bernard, P. S., Herrmann, M. G., Reed, G. H. and Wittwer, C. T. (1999). Rapid *F508del* and *F508C* assay using fluorescent hybridization probes. *Genet. Test* **3**, 365−370.

Gundry, C. N., Vandersteen, J. G., Reed, G. H., Pryor, R. J., Chen, J. and Wittwer, C. T. (2003). Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clin. Chem.* **49**, 396−406.

Gyllensten, U. B. and Erlich, H. A. (1988). Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. *Proc. Natl Acad. Sci. USA* **85**, 7652−7656.

Hackett, N. R., El Sawy, T., Lee, L. Y., Silva, I., O'Leary, J., Rosengart, T. K. and Crystal, R. G. (2000). Use of quantitative TaqMan real-time PCR to track the time-dependent distribution of gene transfer vectors *in vivo*. *Mol. Ther.* **2**, 649−656.

Halford, W. P. (1999). The essential prerequisites for quantitative RT-PCR. *Nat. Biotechnol.* **17**, 835.

Halford, W. P., Falco, V. C., Gebhardt, B. M. and Carr, D. J. J. (1999). The inherent quantitative capacity of the reverse transcription-polymerase chain reaction. *Anal. Biochem.* **266**, 181−191.

Hall, L. L., Bicknell, G. R., Primrose, L., Pringle, J. H., Shaw, J. A. and Furness, P. N. (1998). Reproducibility in the quantification of mRNA levels by RT-PCR-ELISA and RT competitive-PCR-ELISA. *Biotechniques* **24**, 652–657.

Halpin, K., Young, P. L., Field, H. E. and Mackenzie, J. S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J. Gen. Virol.* **81**, 1927–1932.

Hardick, J., Yang, S., Lin, S., Duncan, D. and Gaydos, C. (2003). Use of Roche LightCycler instrument in a real-time PCR for *Trichomonas vaginalis* in urine samples from females and males. *J. Clin. Microbiol.* **41**, 5619–5622.

Härtel, C., Bein, G., Kirchner, H. and Klüter, H. (1999). A human whole-blood assay for analysis of T-cell function by quantification of cytokine mRNA. *Scand. J. Immunol.* **49**, 649–654.

Haugland, R. A., Vesper, S. J. and Wymer, L. J. (1999). Quantitative measurement of *Stachybotrys chartarum conidia* using real time detection of PCR products with the TaqMan (TM) fluorogenic probe system. *Mol. Cell. Probes* **13**, 329–340.

Hawrami, K. and Breur, J. (1999). Development of a fluorogenic polymerase chain reaction assay (TaqMan) for the detection and quantitation of varicella zoster virus. *J. Virol. Meth.* **79**, 33–40.

Hayden, R. T., Uhl, J. R., Qian, X., Hopkins, M. K., Aubry, M. C., Limper, A. H., Lloyd, R. V. and Cockerill, F. R. (2001). Direct detection of *Legionella* species from bronchoalveolar lavage and open lung biopsy specimens: comparison of LightCycler PCR, in situ hybridization, direct fluorescence antigen detection, and culture. *J. Clin. Microbiol.* **37**, 2618–2626.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996). Real time quantitative PCR. *Gen. Meth.* **6**, 986–994.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E. and Wagner, M. (2001a). Comparison of different approaches to quantify *Staphylococcus aureus* by real-time quantitative PCR and application of this techniques for examination of cheese. *Appl. Environ. Microbiol.* **67**, 3122–3126.

Hein, J., Schellenberg, U., Bein, G. and Hackstein, H. (2001b). Quantification of murine IFN-γ mRNA and protein expression: impact of real-time kinetic RT-PCR using SYBR Green I dye. *Scand. J. Immunol.* **54**, 285–291.

Held, T. K., Biel, S. S., Nitsche, A., Kurth, A., Chen, S., Gelderblom, H. R. and Siegert, W. (2000). Treatment of BK virus-associated hemorrhagic cystitis and simultaneous CMV reactivation with cidofovir. *Bone Marrow Transpl.* **26**, 347–350.

Heller, M. J. and Morrison, L. E. (1985). Chemiluminescent and fluorescent probes for DNA hybridization. In *Rapid Detection and Identification of Infectious Agents* (D. T. Kingsbury and S. Falkow, eds), pp. 245–256. Academic Press, New York.

Henderson, D. A., Inglesby, T. V., Bartlett, J. G., Ascher, M. S., Eitzen, E., Jahrling, P. B., Hauer, J., Layton, M., McDade, J., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T., Russell, P. K. and Tonat, K. (1999). Smallpox as a biological weapon: medical and public health management. Working group on civilian biodefense. *JAMA* **281**, 2127–2137.

Henegariu, O., Heerema, N. A., Dloughy, S. R., Vance, G. H. and Vogt, P. H. (1997). Multiplex PCR critical parameters and step-by-step protocol. *Biotechniques* **23**, 504–511.

Hermsen, C. C., Telgt, D. S. C., Linders, E. H. P., van de Locht, L. A. T. F., Eling, W. M. C., Mensink, E. J. B. M. and Sauerwein, R. W. (2001). Detection of *Plasmodium falciparum* malaria parasites in vivo by real-time quantitative PCR. *Mol. Biochem. Parasitol.* **118**, 247–251.

Herrmann, M. G., Dobrowolski, S. F. and Wittwer, C. T. (2000). Rapid β-globin genotyping by multiplexing probe melting temperature and color. *Clin. Chem.* **46**, 425–429.

Higgins, J. A., Ezzell, J., Hinnebusch, B. J., Shipley, M., Henchal, E. A. and Ibrahim, M. S. (1998). 5′ Nuclease PCR assay to detect *Yersinia pestis*. *J. Clin. Microbiol.* **36**, 2284–2288.

Higgins, J. A., Hubalek, Z., Halouzka, J., Elkins, K. L., Sjostedt, A., Shipley, M. and Ibrahim, M. S. (2000). Detection of *Francisella tularensis* in infected mammals and vectors using a probe-based polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **62**, 310–318.

Higgins, J. A., Cooper, M., Schroeder-Tucker, L., Black, S., Miller, D., Karns, K. S., Manthey, E., Breeze, R. and Perdue, M. L. (2003a). A field investigation of *Bacillus anthracis* contamination of U.S. department of agriculture and other Washington, DC, buildings during the anthrax attack of October 2001. *Appl. Environ. Microbiol.* **69**, 593–599.

Higgins, J. A., Nasarabadi, S., Karns, J. S., Shelton, D. R., Cooper, M., Gbakima, A. and Koopman, R. P. (2003b). A handheld real time thermal cycler for bacterial pathogen detection. *Biosens. Bioelectron.* **18**, 1115–1123.

Higuchi, R., Dollinger, G., Walsh, P. S. and Griffith, R. (1992). Simultaneous amplification and detection of specific DNA sequences. *Biotechnology (NY)* **10**, 413–417.

Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993). Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (NY)* **11**, 1026–1030.

Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus*. *Proc. Natl Acad. Sci. USA* **88**, 7276–7280.

Holodniy, M., Katzenstein, D., Sengupta, S., Wang, A. M., Casipit, C., Schwartz, D. H., Konrad, M., Groves, E. and Merigan, T. C. (1991). Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. *J. Infect. Dis.* **163**, 862–866.

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A. and Fach, P. (2003). Making an internal amplification control mandatory for diagnostic PCR. *J. Clin. Microbiol.* **41**, 5835.

Hoshino, Y., Kimura, H., Kuzushima, K., Tsurumi, T., Nemoto, K., Kikuta, A., Nishiyama, Y., Kojima, S., Matsuyama, T. and Morishima, T. (2000). Early intervention in post-transplant lymphoproliferative disorders based on Epstein-Barr viral load. *Bone Marrow Transpl.* **26**, 199–201.

Howell, W. M., Jobs, M. and Brookes, A. J. (2002). iFRET: an improved fluorescence system for DNA-melting analysis. *Genome Res.* **12**, 1401–1407.

Hu, A., Colella, M., Tam, J. S., Rappaport, R. and Cheng, S.-M. (2003). Simultaneous detection, subgrouping, and quantitation of respiratory syncytial virus A and B by real-time PCR. *J. Clin. Microbiol.* **41**, 149–154.

Huang, J., DeGraves, F. J., Gao, D., Feng, P., Schlapp, T. and Kaltenboeck, B. (2001). Quantitative detection of *Chlamydia* spp. by fluorescent PCR in the LightCycler. *Biotechniques* **30**, 150–157.

Hughes, G. J., Smith, J. A., Hanlon, C. A. and Rupprecht, C. E. (2004). Evaluation of a TaqMan PCR assay to detect rabies virus RNA: influence of sequence variation and application to quantification of viral loads. *J. Clin. Microbiol.* **41**, 299–306.

Hurtle, W., Bode, E., Kulesh, D. A., Kaplan, R. S., Garrison, J., Bridge, D., House, M., Frye, M. S., Loveless, B. and Norwood, D. (2004). Detection of *Bacillus anthracis* gyrA gene by using a minor groove binder probe. *J. Clin. Microbiol.* **42**, 179–185.

Hyypiä, T., Puhakka, T., Ruuskanen, O., Mäkelä, M., Arola, A. and Arstila, P. (1998). Molecular diagnosis of human rhinovirus infections: comparison with virus isolation. *J. Clin. Microbiol.* **36**, 2081–2083.

Ibekwe, A. M., Watt, P. M., Grieve, C. M., Sharma, V. K. and Lyons, S. R. (2002). Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. *Appl. Environ. Microbiol.* **68**, 4853–4862.

Ibrahim, M. S., Esposito, J. J., Jahrling, P. B. and Lofts, R. S. (1997). The potential of 5′ nuclease PCR for detecting a single-base polymorphism in *Orthopoxvirus*. *Mol. Cell. Probes* **11**, 143–147.

Ibrahim, M. S., Lofts, R. S., Jahrling, P. B., Henchal, E. A., Weedn, V. W., Northrup, M. A. and Belgrader, P. (1998). Real-time microchip PCR for detecting single-base differences in viral and human DNA. *Anal. Chem.* **70**, 2013–2017.

Ibrahim, M. S., Kulesh, D. A., Saleh, S. S., Damon, I. K., Esposito, J. J., Schmaljohn, A. L. and Jahrling, P. B. (2003). Real-time PCR assay to detect smallpox virus. *J. Clin. Microbiol.* **41**, 3835–3839.

Iriyama, M., Kimura, H., Nishikawa, K., Yoshioka, K., Wakita, T., Nishimura, N., Shibata, M., Ozaki, T. and Morishima, T. (1999). The prevalence of TT virus (TTV) infection and its relationship to hepatitis in children. *Med. Microbiol. Immunol. (Berl.)* **188**, 83–89.

Isacsson, J., Cao, H., Ohlsson, L., Nordgren, S., Svanvik, N., Westman, G., Kubista, M., Sjöback, R. and Sehlstedt, U. (2000). Rapid and specific detection of PCR products using light-up probes. *Mol. Cell. Probes* **14**, 321–328.

Ishiguro, T., Saitoh, J., Yawata, H., Yamagishi, H., Iwasaki, S. and Mitoma, Y. (1995). Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. *Anal. Biochem.* **229**, 207–213.

Jabs, W. J., Hennig, H., Kittel, M., Pethig, K., Smets, F., Bucsky, P., Kirchner, H. and Wagner, H. J. (2001). Normalized quantification by real-time PCR of Epstein-Barr virus load in patients at risk for posttransplant lymphoproliferative disorders. *J. Clin. Microbiol.* **39**, 564–569.

Johnson, J. R. (2000). Development of polymerase chain reaction-based assays for bacterial gene detection. *J. Microbiol. Meth.* **41**, 201–209.

Jordens, J. Z., Lanham, S., Pickett, M. A., Amarasekara, S., Aberywickrema, I. and Watt, P. J. (2000). Amplification with molecular beacon primers and reverse line blotting for the detection and typing of human papillomaviruses. *J. Virol. Meth.* **89**, 29–37.

Josefsson, A., Livak, K. and Gyllensten, U. (1999). Detection and quantitation of human papillomavirus by using the fluorescent 5′ exonuclease assay. *J. Clin. Microbiol.* **37**, 490–496.

Josefsson, A. M., Magnusson, P. K. E., Ylitalo, N., Sørensen, P., Qwarforth-Tubbin, P., Andersen, P. K., Melbye, M., Adami, H.-O. and Gyllensten, U. (2000). Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. *Lancet* **355**, 2189–2193.

Jung, R., Soondrum, K. and Neumaier, M. (2000). Quantitative PCR. *Clin. Chem. Lab. Med.* **38**, 833–836.

Kaneko, S., Murakami, S., Unoura, M. and Kobayashi, K. (1992). Quantitation of hepatitis C virus RNA by competitive polymerase chain reaction. *J. Med. Virol.* **37**, 278–282.

Kares, S., Lönnrot, M., Vuorinen, P., Oikarinen, S., Taurianen, S. and Hyöty, H. (2004). Real-time PCR for rapid diagnosis of entero- and rhinovirus infections using LightCycler. *J. Clin. Virol.* **29**, 99–104.

Kato, T., Mizokami, M., Mukaide, M., Orito, E., Ohno, T., Nakano, T., Tanaka, Y., Kato, H., Sugauchi, F., Ueda, R., Hirashima, N., Shimamatsu, K., Kage, M. and Kojiro, M. (2000). Development of a TT virus DNA quantification system using real-time detection PCR. *J. Clin. Microbiol.* **38**, 94–98.

Kawai, S., Yokosuka, O., Kanda, T., Imazeki, F., Maru, Y. and Saisho, H. (1999). Quantification of hepatitis C virus by TaqMan PCR: comparison with HCV amplicor monitor assay. *J. Med. Virol.* **58**, 121–126.

Ke, D., Ménard, C., Picard, F. J., Boissinot, M., Ouellette, M., Roy, P. H. and Bergeron, M. G. (2000). Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. *Clin. Chem.* **46**, 324–331.

Kearns, A. M., Draper, B., Wipat, W., Turner, A. J. L., Wheeler, J., Freeman, R., Harwood, J., Gould, F. K. and Dark, J. H. (2001a). LightCycler-based quantitative PCR for detection of cytomegalovirus in blood, urine and respiratory samples. *J. Clin. Microbiol.* **39**, 2364–2365.

Kearns, A. M., Guiver, M., James, V. and King, J. (2001b). Development and evaluation of a real-time quantitative PCR for the detection of human cytomegalovirus. *J. Virol. Meth.* **95**, 121–131.

Kearns, A. M., Turner, A. J. L., Taylor, C. E., George, P. W., Freeman, R. and Gennery, A. R. (2001c). LightCycler-based quantitative PCR for rapid detection of human herpesvirus 6 DNA in clinical material. *J. Clin. Microbiol.* **39**, 3020–3021.

Kehl, S. C., Henrickson, K. J., Hua, W. and Fan, J. (2001). Evaluation of the hexaplex assay for detection of respiratory viruses in children. *J. Clin. Microbiol.* **39**, 1696–1701.

Keller, G. H., Huang, D.-P., Shih, J. W.-K. and Manak, M. M. (1990). Detection of hepatitis B virus DNA in serum by polymerase chain reaction amplification and microtiter sandwich hybridization. *J. Clin. Microbiol.* **28**, 1411–1416.

Kemp, D. J., Churchill, M. J., Smith, D. B., Biggs, B. A., Foote, S. J., Peterson, M. G., Samaras, N., Deacon, N. J. and Doherty, R. (1990). Simplified colorimetric analysis of polymerase chain reactions: detection of HIV sequences in AIDS patients. *Gene* **94**, 223–228.

Kennedy, M. M., Lucas, S. B., Jones, R. R., Howells, D. D., Picton, S. J., Hanks, E. E., McGee, J. O. and O'Leary, J. (1997a). HHV8 and Kaposi's sarcoma: a time cohort study. *J. Clin. Pathol.* **50**, 96–100.

Kennedy, M. M., Lucas, S. B., Russell-Jones, R., Howells, D. D., Picton, S. J., Bardon, A., Comley, I. L., McGee, J. O. and O'Leary, J. J. (1997b). HHV8 and female Kaposi's sarcoma. *J. Pathol.* **183**, 447–452.

Kennedy, M. M., Cooper, K., Howells, D. D., Picton, S., Biddolph, S., Lucas, S. B., McGee, J. O. and O'Leary, J. J. (1998a). Identification of HHV8 in early Kaposi's sarcoma: implications for Kaposi's sarcoma pathogenesis. *J. Clin. Pathol.* **51**, 14–20.

Kennedy, M. M., O'Leary, J. J., Oates, J. L., Lucas, S. B., Howells, D. D., Picton, S. and McGee, J. O. (1998b). Human herpes virus 8 (HHV-8) in Kaposi's sarcoma: lack of association with Bcl-2 and p53 protein expression. *J. Clin. Pathol.* **51**, 155–159.

Kennedy, M. M., Biddolph, S., Lucas, S. B., Howells, D. D., Picton, S., McGee, J. O. and O'Leary, J. J. (1999a). CD40 upregulation is independent of HHV-8 in the pathogenesis of Kaposi's sarcoma. *J. Clin. Pathol.* **52**, 32–36.

Kennedy, M. M., Biddolph, S., Lucas, S. B., Howells, D. D., Picton, S., McGee, J. O., Silva, I., Uhlmann, V., Luttich, K. and O'Leary, J. (1999b). Cyclin D1 expression and HHV-8 in Kaposi sarcoma. *J. Clin. Pathol.* **52**, 569–573.

Kidd, I. M., Clark, D. A. and Emery, V. C. (2000). A non-radioisotopic quantitative competitive polymerase chain reaction method: application in measurement of human herpesvirus 7 load. *J. Virol. Meth.* **87**, 177–181.

Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R. and Lens, P. (1991). NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. *J. Virol. Meth.* **35**, 273–286.

Kikuchi, T., Iwasaki, K., Nishihara, H., Takamura, Y. and Yagi, O. (2002). Quantitative and rapid detection of the trichloroethylene-degrading bacterium *Methylocystis* sp. in groundwater by real-time PCR. *Appl. Microbiol. Biotechnol.* **59**, 731–736.

Kilpatrick, D. R., Nottay, B., Yang, C. F., Yang, S. J., Mulders, M. N., Holloway, B. P., Pallansch, M. A. and Kew, O. M. (1996). Group-specific identification of polioviruses by PCR using primers containing mixed-base or deoxyinosine residue at positions of codon degeneracy. *J. Clin. Microbiol.* **34**, 2990–2996.

Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T. and Morishima, T. (1999). Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J. Clin. Microbiol.* **37**, 132–136.

Klaschik, S., Lehmann, L. E., Raadts, A., Book, M., Hoeft, A. and Stuber, F. (2002). Real-time PCR for the detection and differentiation of Gram-positive and Gram-negative bacteria. *J. Clin. Microbiol.* **40**, 4304–4307.

Klein, D. (2002). Quantification using real-time PCR technology: applications and limitations. *Trends Mol. Med.* **8**, 257–260.

Klein, D., Janda, P., Steinborn, R., Salmons, B. and Günzburg, W. H. (1999). Proviral load determination of different feline immunodeficiency virus isolates using real-time polymerase chain reaction: Influence of mismatches on quantification. *Electrophoresis* **20**, 291–299.

Komurian-Pradel, F., Paranhos-Baccalà, G., Sodoyer, M., Chevallier, P., Mandrand, B., Lotteau, V. and André, P. (2001). Quantitation of HCV RNA using real-time PCR and fluorimetry. *J. Virol. Meth.* **95**, 111–119.

Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., Olsen, C. E. and Wengel, J. (1998). LNA (locked nuciec acids): synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclo-nucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron* **54**, 3607–3630.

Kox, L. F. F., Noordhoek, G. T., Kunakorn, M., Mulder, S., Sterrenburg, M. and Kolk, A. H. J. (1996). Microwell hybridization assay for detection of PCR products from *Mycobacterium tuberculosis* complex and the recombinant *Mycobacterium smegmatis* strain 1008 used as an internal control. *J. Clin. Microbiol.* **34**, 2117–2120.

Kraus, G., Cleary, T., Miller, N., Seivright, R., Young, A. K., Spruill, G. and Hnatyszyn, H. J. (2001). Rapid and specific detection of the *Mycobacterium tuberculosis* complex using fluorogenic probes and real-time PCR. *Mol. Cell. Probes* **15**, 375–383.

Kreuzer, K.-A., Bohn, A., Lass, U., Peters, U. R. and Schmidt, C. A. (2000). Influence of DNA polymerases on quantitative PCR results using TaqMan probe format in the LightCycler instrument. *Mol. Cell. Probes* **14**, 57–60.

Kruse, N., Pette, M., Toyka, K. and Rieckmann, P. (1997). Quantification of cytokine mRNA expression by RT PCR in samples of previously frozen blood. *J. Immunol. Meth.* **210**, 195–203.

Kumar, R., Singh, S. K., Koshkin, A. A., Rajwanshi, V. K., Meldgaard, M. and Wengel, J. (1998). The first analogues of LNA (locked nucleic acids): phosphorothioate-LNA and 2′-thio-LNA. *Bioorg. Med. Chem. Lett.* **8**, 2219–2222.

Kupferschmidt, O., Krüger, D., Held, T. K., Ellerbrok, H., Siegert, W. and Janitschke, K. (2001). Quantitative detection of *Toxoplasma gondii* DNA in human

body fluids by TaqMan polymerase chain reaction. *Clin. Microbiol. Infect.* **7**, 120–124.

Kurata, S., Kanagawa, T., Yamada, K., Torimura, M., Yokomaku, T., Kamagata, Y. and Kurane, R. (2001). Fluorescent quenching-based quantitative detection of specific DNA/RNA using a BODIPY® FL-labeled probe or primer. *Nucleic Acids Res.* **29**, e34.

Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B. and Hedgpeth, J. (2000). 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res.* **28**, 655–661.

Lai, K. K.-Y., Cook, L., Wendt, S., Corey, L. and Jerome, K. R. (2003). Evaluation of real-time PCR versus PCR with liquid-phase hybridization for detection of enterovirus RNA in cerebrospinal fluid. *J. Clin. Microbiol.* **41**, 3133–3141.

Lallemand, F., Desire, N., Rozenbaum, W., Nicolas, J.-C. and Marechal, V. (2000). Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. *J. Clin. Microbiol.* **38**, 1404–1408.

Lanciotti, R. S. and Kerst, A. J. (2001). Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis Encephalitis. *J. Clin. Microbiol.* **39**, 4506–4513.

Lanciotti, R. S., Kerst, A. J., Nasci, R. S., Godsey, M. S., Mitchell, C. J., Savage, H. M., Komar, N., Panella, N. A., Allen, B. C., Volpe, K. E., Davis, B. S. and Roehrig, J. T. (2000). Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J. Clin. Microbiol.* **38**, 4066–4071.

Landt, O. (2001). Selection of hybridisation probes for real-time quantification and genetic analysis. In *Rapid Cycle Real-Time PCR: Methods and Applications* (S. Meuer, C. Wittwer and K. Nakagawara, eds), pp. 35–41. Springer, Germany.

Lanham, S., Herbert, A. and Watt, P. (2001). HPV detection and measurement of HPV-16, telomerase, and survivin transcripts in colposcopy clinic patients. *J. Clin. Pathol.* **54**, 304–308.

Laue, T., Emmerich, P. and Schmitz, H. (1999). Detection of dengue virus RNA inpatients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J. Clin. Microbiol.* **37**, 2543–2547.

Lay, M. J. and Wittwer, C. T. (1997). Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clin. Chem.* **43**, 2262–2267.

Lee, L. G., Connell, C. R. and Bloch, W. (1993). Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res.* **21**, 3761–3766.

Lee, L. G., Livak, K. J., Mullah, B., Graham, R. J., Vinayak, R. S. and Woudenberg, T. M. (1999a). Seven-color, homogeneous detection of six PCR products. *Biotechniques* **27**, 342–349.

Lee, M. A., Brightwell, G., Leslie, D., Bird, H. and Hamilton, A. (1999b). Fluorescent detection techniques for real-time multiplex strand specific detection of *Bacillus anthracis* using rapid PCR. *J. Appl. Microbiol.* **87**, 218–223.

Lee, M.-A., Tan, C.-H., Aw, L.-T., Tang, C.-S., Singh, M., Lee, S.-H., Chia, H.-P. and Yap, E. P. H. (2002a). Real-time fluorescence-based PCR for detection of malaria parasites. *J. Clin. Microbiol.* **40**, 4343–4345.

Lee, M. A., Siddle, A. L. and Page, R. H. (2002b). ResonSense®: simple linear fluorescent probes for quantitative homogeneous rapid polymerase chain reaction. *Anal. Chim. Acta* **457**, 61–70.

Lefevre, J., Hankins, C., Pourreaux, K., Voyer, H. and Coutlée, F. (2003). Real-time PCR assays using internal controls for quantitation of HPV-16 and β-globin DNA in cervicovaginal lavages. *J. Virol. Meth.* **114**, 135–144.

Leutenegger, C. M., Klein, D., Hofmann-Lehmann, R., Mislin, C., Hummel, U., Boni, J., Boretti, F., Guenzburg, W. H. and Lutz, H. (1999). Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. *J. Virol. Meth.* **78**, 105–116.

Lew, A. E., Bock, R. E., Miles, J., Cuttell, L. B., Steer, P. and Nadin-Davis, S. A. (2004a). Sensitive and specific detection of bovine immunodeficiency virus and bovine syncytial virus by 5′ Taq nuclease assays with fluorescent 3′ minor groove binder-DNA probes. *J. Virol. Meth.* **116**, 1–9.

Lew, A. E., Bock, R. E., Molloy, J. B., Minchin, C. M., Robinson, S. J. and Steer, P. (2004b). Sensitive and specific detection of proviral bovine leukemia virus by 5′ Taq nuclease PCR using a 3′ minor groove binder fluorogenic probe. *J. Virol. Meth.* **115**, 167–175.

Lewin, S. R., Vesanen, M., Kostrikis, L., Hurley, A., Duran, M., Zhang, L., Ho, D. D. and Markowitz, M. (1999). Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus type 1-infected individuals on prolonged effective antiretroviral therapy. *J. Virol.* **73**, 6099–6103.

Lewis-Jones, S. (2002). The zoonotic poxviruses. *Dermatol. Nurs.* **14**, 79–82.

Li, Q.-G., Liang, J.-X., Luan, G.-Y., Zhang, Y. and Wang, K. (2000). Molecular beacon-based homogeneous fluorescence PCR assay for the diagnosis of infectious diseases. *Anal. Sci.* **16**, 245–249.

Li, Q., Luan, G., Guo, Q. and Liang, J. (2002). A new class of homogeneous nucleic acid probes based on specific displacement hybridization. *Nucleic Acids Res.* **30**, e5.

Limaye, A. P., Jerome, K. R., Kuhr, C. S., Ferrenberg, J., Huang, M.-L., Davis, C. L., Corey, L. and Marsh, C. L. (2000). Quantitation of BK virus load in serum for the diagnosis of BK virus-associated nephropathy in renal transplant recipients. *J. Infect. Dis.* **183**, 1669–1672.

Limaye, A. P., Huang, M.-L., Leisenring, W., Stensland, L., Corey, L. and Boeckh, M. (2001). Cytomegalovirus (CMV) DNA load in plasma for the diagnosis of CMV disease before engraftment in hematopoietic stem-cell transplant recipients. *J. Infect. Dis.* **183**, 377–382.

Lindler, L. E. and Fan, W. (2003). Development of a 5′ nuclease assay to detect ciprofloxacin resistant isolates of the biowarfare agent *Yersinia pestis*. *Mol. Cell. Probes* **17**, 41–47.

Lindler, L. E., Fan, W. and Jahan, N. (2001). Detection of ciprofloxacin-resistant *Yersinia pestis* by fluorogenic PCR using the LightCycler. *J. Clin. Microbiol.* **39**, 3649–3655.

Lipson, S. M. (2002). Rapid laboratory diagnostics during the winter respiratory virus season. *J. Clin. Microbiol.* **40**, 733–734.

Liss, B. (2002). Improved quantitative real-time RT-PCR for expression profiling of individual cells. *Nucleic Acids Res.* **30**, e89.

Liu Yin, J. A. (2002). Minimal residual disease in acute myeloid leukaemia. *Best Pract. Res. Clin. Haematol.* **15**, 119–135.

Liu, W. and Saint, D. A. (2002). A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Anal. Biochem.* **302**, 52–59.

Real-time Fluorescent PCR Techniques to Study

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2_t^{-\Delta\Delta C}$ method. *Methods* **25**, 402–408.

Livak, K. J., Flood, S. J. A., Marmaro, J., Giusti, W. and Deetz, K. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Meth. Appl.* **4**, 357–362.

Lo, Y. M. D., Chan, L. Y. S., Lo, K.-W., Leung, S.-F., Zhang, J., Chan, A. T. C., Lee, J. C. K., Hjelm, N. M., Johnson, P. J. and Huang, D. P. (1999). Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res.* **59**, 1188–1191.

Locatelli, G., Santoro, F., Veglia, F., Gobbi, A., Lusso, P. and Malnati, M. S. (2000). Real-time quantitative PCR for human herpesvirus 6 DNA. *J. Clin. Microbiol.* **38**, 4042–4048.

Lockhart, D. J. and Winzeler, E. A. (2000). Genomics, gene expression and DNA arrays. *Nature* **405**, 827–836.

Lomeli, H., Tyagi, S., Pritchard, C. G., Lizardi, P. M. and Kramer, F. R. (1989). Quantitative assays based on the use of replicatable hybridization probes. *Clin. Chem.* **35**, 1826–1831.

Loparev, V. N., McCaustland, K., Holloway, B. P., Krause, P. R., Takayama, M. and Schmid, D. S. (2000). Rapid genotyping of varicella-zoster virus vaccine and wild-type strains with fluorophore-labeled hybridization probes. *J. Clin. Microbiol.* **38**, 4315–4319.

Löseke, S., Grage-Griebenow, E., Wagner, A., Gehlhar, K. and Bufe, A. (2003). Differential expression of IFN-α subtypes in human PBMC: evaluation of novel real-time PCR assays. *J. Immunol. Meth.* **276**, 207–222.

Lucchini, S., Thompson, A. and Hinton, J. C. D. (2001). Microarrays for microbiologists. *Microbiology* **147**, 1403–1414.

Lunge, V. R., Miller, B. J., Livak, K. J. and Batt, C. A. (2002). Factors affecting the performance of 5′ nuclease PCR assays for *Listeria monocytogenes* detection. *J. Microbiol. Meth.* **51**, 361–368.

Luria, S. E. and Darnell, J. E. (1967). Titration of viruses. In *General Virology* (S. E. Luria and J. E. Darnell, eds), 2nd edn., pp. 35–49. New York, Wiley.

Lyamichev, V., Brow, M. A. D. and Dahlberg, J. E. (1993). Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. *Science* **260**, 778–783.

Machida, U., Kami, M., Fukui, T., Kazuyama, Y., Kinoshita, M., Tanaka, Y., Kanda, Y., Ogawa, S., Honda, H., Chiba, S., Mitani, K., Muto, Y., Osumi, K., Kimura, S. and Hirai, H. (2000). Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation. *J. Clin. Microbiol.* **38**, 1536–1542.

Mackay, I. M., Metharom, P., Sloots, T. P. and Wei, M. Q. (2001). Quantitative PCR-ELAHA for the determination of retroviral vector transduction efficiency. *Mol. Ther.* **3**, 801–807.

Mackay, I. M., Arden, K. E. and Nitsche, A. (2002). Real-time PCR in virology. *Nucleic Acids Res.* **30**, 1292–1305.

Mackay, I. M., Gardam, T., Arden, K. E., McHardy, S., Whiley, D. M., Crisante, E. and Sloots, T. P. (2003a). Co-detection and discrimination of six human herpesviruses by multiplex PCR-ELAHA. *J. Clin. Virol.* **28**, 291–302.

Mackay, I. M., Jacob, K. C., Woolhouse, D., Waller, K., Syrmis, M. W., Whiley, D. M., Siebert, D. J., Nissen, M. N. and Sloots, T. P. (2003b). Molecular assays for the detection of human metapneumovirus. *J. Clin. Microbiol.* **41**, 100–105.

MacKenzie, J., Gallagher, A., Clayton, R. A., Perry, J., Eden, O. B., Ford, A. M., Greaves, M. F. and Jarrett, R. F. (2001). Screening for herpesvirus genomes in common acute lymphoblastic leukemia. *Leukemia* **15**, 415–421.

Makino, S.-I., Cheun, H. I., Watarai, M., Uchida, I. and Takeshi, K. (2001). Detection of anthrax spores from the air by real-time PCR. *Lett. Appl. Microbiol.* **33**, 237–240.

Martell, M., Gómez, J., Esteban, J. I., Sauleda, S., Quer, J., Cabot, B., Esteban, R. and Guardia, J. (1999). High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J. Clin. Microbiol.* **37**, 327–332.

Martineau, F., Picard, F. J., Lansac, N., Ménard, C., Roy, P. H., Ouellette, M. and Bergeron, M. G. (2000). Correlation between resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob. Agent Chem.* **44**, 231–238.

Matsumura, M., Hikiba, Y., Ogura, K., Togo, G., Tsukuda, I., Ushikawa, K., Shiratori, Y. and Omata, M. (2001). Rapid detection of mutations in the 23S rRNA gene of *Helicobacter pylori* that confers resistance to clarithromycin treatment to the bacterium. *J. Clin. Microbiol.* **39**, 691–695.

Matthews, J. A. and Kricka, L. J. (1988). Analytical strategies for the use of DNA probes. *Anal. Biochem.* **169**, 1–25.

McCartney, H. A., Foster, S. J., Fraaije, B. A. and Ward, E. (2003). Molecular diagnostics for fungal plant pathogens. *Pest Manag. Sci.* **59**, 129–142.

McCreedy, B. J. and Callaway, T. H. (1993). Laboratory design and work flow. In *Diagnostic Molecular Microbiology: Principles and Applications* (D. H. Persing, T. F. Smith, F. C. Tenover and T. J. White, eds), pp. 149–159. ASM, Washington, DC.

Menzo, S., Bagnarelli, P., Giacca, M., Manzin, A., Varaldo, P. E. and Clementi, M. (1992). Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. *J. Clin. Microbiol.* **30**, 1752–1757.

Mhlanga, M. M. and Malmberg, L. (2001). Using molecular beacons to detect single-nucleotide polymorphisms with real-time PCR. *Methods* **25**, 463–471.

Miller, N., Cleary, T., Kraus, G., Young, A. K., Spruill, G. and Hnatyszyn, H. J. (2002). Rapid and specific detection of *Mycobacterium tuberculosis* from acid-fast bacillus smear-positive respiratory specimens and BacT/ALERT MP culture bottles by using fluorogenic probes and real time-PCR. *J. Clin. Microbiol.* **40**, 4143–4147.

Mitchell, T. J. (1998). Molecular basis of virulence. *Arch. Dis. Child.* **78**, 197–209.

Monopoeho, S., Mignotte, B., Schwartzbrod, L., Marechal, V., Nicolas, J.-C., Billaudel, S. and Férré, V. (2000). Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *Biotechniques* **29**, 88–93.

Monpoeho, S., Coste-Burel, M., Costa-Mattioli, M., Besse, B., Chomel, J. J., Billaudel, S. and Ferré, V. (2002). Application of a real-time polymerase chain reaction with internal positive control for detection and quantification of enterovirus in cerebrospinal fluid. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**, 532–536.

Moody, A., Sellers, S. and Bumstead, N. (2000). Measuring infectious bursal disease virus RNA in blood by multiplex real-time quantitative RT-PCR. *J. Virol. Meth.* **85**, 55–64.

Möller, A. and Jansson, J. K. (1997). Quantification of genetically tagged cyanobacteria in Baltic sea sediment by competitive PCR. *Biotechniques* **22**, 512–518.

Morrison, L. E., Halder, T. C. and Stols, L. M. (1989). Solution-phase detection of polynucleotides using interacting fluorescent labels and competitive hybridization. *Anal. Biochem.* **183**, 231–244.

Morrison, T. B., Weis, J. J. and Wittwer, C. T. (1998a). Quantification of low-copy transcripts by continuous SYBR® green I monitoring during amplification. *Biotechniques* **24**, 954–962.

Morrison, T. M., Weis, J. J. and Wittwer, C. T. (1998b). Quantification of low-copy transcripts by continuous SYBR green I monitoring during amplification. *Biotechniques* **24**, 954–962.

Mothershed, E. A., Sacchi, C. T., Whitney, A. M., Barnett, G. A., Ajello, G. W., Schmink, S., Mayer, L. W., Phelan, M., Taylor, T. H., Bernhardt, S. A., Rosenstein, N. E. and Popovic, T. (2004). Use of real-time PCR to resolve slide agglutination discrepancies in serogroup identification of *Neisseria meningitidis*. *J. Clin. Microbiol.* **42**, 320–328.

Mullis, K. B. and Faloona, F. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Meth. Enzymol.* **155**, 335–350.

Muti, G., Klersy, C., Baldanti, F., Granata, S., Oreste, P., Pezzetti, L., Gatti, M., Gargantini, L., Caramella, M., Mancini, V., Gerna, G. and Morra, E. (2003). Epstein-Barr virus (EBV) load and interleukin-10 in EBV-positive and EBV-negative post-transplant lymphoproliferative disorders. *Br. J. Haem.* **122**, 927–933.

Najioullah, F., Thouvenot, D. and Lina, B. (2001). Development of a real-time PCR procedure including an internal control for the measurement of HCMV viral load. *J. Virol. Meth.* **92**, 55–64.

Nakayama, H., Yokoi, H. and Fujita, J. (1992). Quantification of mRNA by non-radioactive RT-PCR and CCD imaging system. *Nucleic Acids Res.* **20**, 4939.

Natarajan, V., Plishka, R. J., Scott, E. W., Lane, H. C. and Salzman, N. P. (1994). An internally controlled virion PCR for the measurement of HIV-1 RNA in plasma. *PCR Meth. Appl.* **3**, 346–350.

Nazarenko, I. A., Bhatnager, S. K. and Hohman, R. J. (1997). A closed tube format for amplification and detection of DNA based on energy transfer. *Nucleic Acids Res.* **25**, 2516–2521.

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D. and Raschtchian, A. (2002). Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res.* **30**, e37.

Ng, L. F. P., Wong, M., Koh, S., Ooi, E.-E., Tang, K.-F., Leong, H.-N., Ling, A.-E., Agathe, L. V., Tan, J., Liu, E. T., Ren, E.-C., Ng, L.-C. and Hibberd, M. L. (2004). Detection of severe acute respiratory syndrome coronavirus in blood of infected patients. *J. Clin. Microbiol.* **42**, 347–350.

Nicoll, S., Brass, A. and Cubie, H. A. (2001). Detection of herpes viruses in clinical samples using real-time PCR. *J. Virol. Meth.* **96**, 25–31.

Niesters, H. G. M. (2001). Quantitation of viral load using real-time amplification techniques. *Methods* **25**, 419–429.

Niesters, H. G. M. (2002). Clinical virology in real time. *J. Clin. Virol.* **25**, S3–S12.

Niesters, H. G., van Esser, J., Fries, E., Wolthers, K. C., Cornelissen, J. and Osterhaus, A. D. (2000). Development of a real-time quantitative assay for detection of Epstein-Barr virus. *J. Clin. Microbiol.* **38**, 712–715.

Nijhuis, M., van Maarseveen, N., Schuurman, R., Verkuijlen, S., de Vos, M., Hendricksen, K. and van Loon, A. M. (2002). Rapid and sensitive routine detection of all members of the genus *Enterovirus* in different clinical specimens by real-time PCR. *J. Clin. Microbiol.* **40**, 3666–3670.

Nitsche, A., Steuer, N., Schmidt, C. A., Landt, O. and Siegert, W. (1999). Different real-time PCR formats compared for the quantitative detection of human cytomegalovirus DNA. *Clin. Chem.* **45**, 1932–1937.

Nitsche, A., Steuer, N., Schmidt, C. A., Landt, O., Ellerbrok, H., Pauli, G. and Siegert, W. (2000). Detection of human cytomegalovirus DNA by real-time quantitative PCR. *J. Clin. Microbiol.* **38**, 2734–2737.

Nitsche, A., Ellerbrok, H. and Pauli, G. (2004). Detection of orthopoxvirus DNA by real-time PCR and identification of variola virus DNA by melting analysis. *J. Clin. Microbiol.* **42**, 1207–1213.

Nogva, H. K., Rudi, K., Naterstad, K., Holck, A. and Lillehaug, D. (2000). Application of 5′-nuclease PCR for quantitative detection of *Listeria* monocytogenes in pure cultures, water, skim milk and unpasteurized whole milk. *Appl. Environ. Microbiol.* **66**, 4266–4271.

Northrup, M. A. (1998). A miniature analytical instrument for nucleic acids based on micromachined silicon reaction chambers. *Anal. Chem.* **70**, 918–922.

Nulens, E. and Voss, A. (2002). Laboratory diagnosis and biosafety issues of biological warfare agents. *Clin. Microbiol. Infect.* **8**, 455–466.

Nutiu, R. and Li, Y. (2002). Tripartite molecular beacons. *Nucleic Acids Res.* **30**, e94.

Obika, S., Nanbu, D., Hari, Y., Andoh, J.-I., Morio, K.-I., Doi, T. and Imanishi, T. (1998). Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2′-O,4′-C-methyleneribonucleosides. *Tetrahedron Lett.* **39**, 5401–5404.

Oggioni, M. R., Meacci, F., Carattoli, A., Ciervo, A., Orru, G., Cassone, A. and Pozzi, G. (2002). Protocol for real-time PCR inhibition of anthrax spores from nasal swabs after broth enrichment. *J. Clin. Microbiol.* **40**, 3956–3963.

Ogilvie, M. (2001). Molecular techniques should not now replace cell culture in diagnostic virology laboratories. *Rev. Med. Virol.* **11**, 351–354.

Ohyashiki, J. K., Suzuki, A., Aritaki, K., Nagate, A., Shoji, N., Ohyashiki, K., Ojima, T., Abe, K. and Yamamoto, K. (2000). Use of real-time PCR to monitor human herpesvirus 6 reactivation after allogeneic bone marrow transplantation. *Int. J. Mol. Med.* **6**, 427–432.

O'Leary, J., Kennedy, M., Howells, D., Silva, I., Uhlmann, V., Luttich, K., Biddolph, S., Lucas, S., Russell, J., Bermingham, N., O'Donovan, M., Ring, M., Kenny, C., Sweeney, M., Sheils, O., Martin, C., Picton, S. and Gatter, K. (2000a). Cellular localisation of HHV-8 in Castleman's disease: is there a link with lymph node vascularity? *J. Clin. Pathol.* **53**, 69–76.

O'Leary, J. J., Kennedy, M., Luttich, K., Uhlmann, V., Silva, I., Russell, J., Sheils, O., Ring, M., Sweeney, M., Kenny, C., Bermingham, N., Martin, C., O'Donovan, M., Howells, D., Picton, S. and Lucas, S. B. (2000b). Localisation of HHV-8 in AIDS related lymphadenopathy. *J. Clin. Pathol.* **53**, 43–47.

O'Mahony, J. and Hill, C. (2002). A real time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR green and the Light Cycler. *J. Microbiol. Meth.* **51**, 283–293.

Orlando, C., Pinzani, P. and Pazzagli, M. (1998). Developments in quantitative PCR. *Clin. Chem. Lab. Med.* **36**, 255–269.

O'Sullivan, C. E., Kasai, M., Francesconi, A., Petraitis, V., Petraitiene, R., Kelaher, A. M., Sarafandi, A. A. and Walsh, T. J. (2003). Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. *J. Clin. Microbiol.* **41**, 5676–5682.

Overbergh, L., Valckx, D., Waer, M. and Mathieu, C. (1999). Quantification of murine cytokine mRNAs using real-time quantitative reverse transcriptase PCR. *Cytokine* **11**, 305–312.

Overbergh, L., Giulietti, A., Valckx, D., Decallonne, B., Bouillon, R. and Mathieu, C. (2003). The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J. Biomol. Tech.* **14**, 33–43.

Real-time Fluorescent PCR Techniques to Study

Pahl, A., Kühlbrandt, U., Brune, K., Röllinghoff, M. and Gessner, A. (1999). Quantitative detection of *Borrelia burgdorferi* by real-time PCR. *J. Clin. Microbiol.* **37**, 1958–1963.

Pang, X. L. (2004). Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. *J. Med. Virol.* **72**, 496–501.

Peiris, J. S., Lai, S. T., Poon, L. L., Guan, Y., Yam, L. Y., Lim, W., Nicholls, J., Yee, W. K., Yan, W. W., Cheung, M. T., Cheng, V. C., Chan, K. H., Tsang, D. N., Yung, R. W., Ng, T. K., Yuen, K. Y. and SARS study group (2003). Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* **361**, 1319–1325.

Persing, D. H. (1993). *In vitro* nucleic acid amplification techniques. In *Diagnostic Molecular Microbiology: Principles and Applications* (D. H. Persing, T. F. Smith, F. C. Tenover and T. J. White, eds), pp. 51–87. ASM, Washington, DC.

Peter, J. B. and Sevall, J. S. (2001). Review of 3200 serially received CSF samples submitted for type-specific HSV detection by PCR in the reference laboratory setting. *Mol. Cell. Probes* **15**, 177–182.

Petersen, M. and Wengel, J. (2003). LNA: a versatile tool for therapeutics and genomics. *Trends Biotechnol.* **21**, 74–81.

Petrik, J., Pearson, G. J. M. and Allain, J.-P. (1997). High throughput PCR detection of HCV based on semiautomated multisample RNA capture. *J. Virol. Meth.* **64**, 147–159.

Pfaffl, M. W., Horgan, G. W. and Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.

Pfaller, M. A. (1999). Molecular epidemiology in the care of patients. *Arch. Pathol. Lab. Med.* **123**, 1007–1010.

PhorTech. Rapid rise in platforms for realtime PCR transforms DNA amplification instrumentation market. http://www.phortech.com/02ampi.htm, 2003.

Pietilä, J., He, Q., Oksi, J. and Viljanen, M. K. (2000). Rapid differentiation of *Borrelia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi* sensu stricto by LightCycler fluorescence melting curve analysis of a PCR product of the recA gene. *J. Clin. Microbiol.* **38**, 2756–2759.

Pinzani, P., Bonciani, L., Pazzagli, M., Orlando, C., Guerrini, S. and Granchi, L. (2004). Rapid detection of *Oenococcus oeni* in wine by real-time quantitative PCR. *Lett. Appl. Microbiol.* **38**, 118–124.

Poutanen, S. M., Low, D. E., Henry, B., Finkelstein, S., Rose, D., Green, K., Tellier, R., Draker, R., Adachi, D., Ayers, M., Chan, A. K., Skowronski, D. M., Salit, I., Simor, A. E., Slutsky, A. S., Doyle, P. W., Krajden, M., Petric, M., Brunham, R. C. and McGeer, A. J. (2003). Identification of severe acute respiratory syndrome in Canada. *N. Engl. J. Med.* **348**, 1995–2005.

Probert, W. S., Bystrom, S. L., Khashe, S., Schrader, K. N. and Wong, J. D. (2002). 5′ Exonuclease assay for detection of serogroup Y *Neisseria meningitidis*. *J. Clin. Microbiol.* **40**, 4325–4328.

Qi, Y., Patra, G., Liang, X., Williams, L. E., Rose, S., Redkar, R. J. and del Vecchio, V. G. (2001). Utilization of the rpoB gene as a specific chromosomal marker for real-time PCR detection of *Bacillus anthracis*. *Appl. Environ. Microbiol.* **67**, 3720–3727.

Quereda, C., Corral, I., Laguna, F., Valencia, M. E., Tenorio, A., Echevarria, J. E., Navas, E., Martín-Davila, P., Moreno, A., Moreno, V., Gonzalez-Lahoz, J. M., Arribas, J. R. and Guerrero, A. (2000). Diagnostic utility of a multiplex herpesvirus PCR assay performed with cerebrospinal fluid from human immunodeficiency virus-infected patients with neurological disorders. *J. Clin. Microbiol.* **38**, 3061–3067.

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W. and Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **313**, 856−862.

Randegger, C. C. and Hachler, H. (2001). Real-time PCR and melting curve analysis for reliable and rapid detection of SHV extended-spectrum β-lactamases. *Antimicrob. Agent Chem.* **45**, 1730−1736.

Ratge, D., Scheiblhuber, B., Landt, O., Berg, J. and Knabbe, C. (2002). Two-round rapid-cycle RT-PCR in single closed capillaries increases the sensitivity of HCV RNA detection and avoids amplicon carry-over. *J. Clin. Virol.* **24**, 161−172.

Rauter, C., Oehme, R., Diterich, I., Engele, M. and Hartung, T. (2002). Distribution of clinically relevant *Borrelia* genospecies in ticks assessed by a novel, single-run, real-time PCR. *J. Clin. Microbiol.* **40**, 36−43.

Read, S. J. (2001). Recovery efficiencies of nucleic acid extraction kits as measured by quantitative LightCycler™ PCR. *J. Clin. Pathol.* **54**, 86−90.

Read, S. J., Mitchell, J. L. and Fink, C. G. (2001). LightCycler multiplex PCR for the laboratory diagnosis of common viral infections of the central nervous system. *J. Clin. Microbiol.* **39**, 3056−3059.

Reid, S. M., Gerris, N. P., Hutchings, G. H., King, D. P. and Alexandersen, S. (2004). Evaluation of real-time reverse transcription polymerase chain reaction assays for the detection of swine vesicular disease virus. *J. Virol. Meth.* **116**, 169−176.

Reischl, U., Linde, H.-J., Metz, M., Leppmeier, B. and Lehn, N. (2000). Rapid identification of methicillin-resistant *Staphylococcus aureus* and simultaneous species confirmation using real-time fluorescence PCR. *J. Clin. Microbiol.* **38**, 2429−2433.

Reischl, U., Linde, H.-J., Lehn, N., Landt, O., Barratt, K. and Wellinghausen, N. (2002). Direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila* in clinical specimens by dual-colour real-time PCR and melting curve analysis. *J. Clin. Microbiol.* **40**, 3814−3817.

Reiss, R. A. and Rutz, B. (1999). Quality control PCR: a method for detecting inhibitors of *Taq* DNA polymerase. *Biotechniques* **27**, 920−926.

Ririe, K. M., Rasmussen, R. P. and Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal. Biochem.* **245**, 154−160.

Roberts, T. C., Brennan, D. C., Buller, R. S., Gaudreault-Keener, M., Schnitzler, M. A., Sternhell, K. E., Garlock, K. A., Singer, G. G. and Storch, G. A. (1998). Quantitative polymerase chain reaction to predict occurrence of symptomatic cytomegalovirus infection and assess response to ganciclovir therapy in renal transplant recipients. *J. Infect. Dis.* **178**, 626−636.

Rodrigo, A. G., Goracke, P. C., Rowhanian, K. and Mullins, J. I. (1997). Quantitation of target molecules from polymerase chain reaction-based limiting dilution assays. *AIDS Res. Hum. Retro.* **13**, 737−742.

Roe, J. D., Haugland, R. A., Vesper, S. J. and Wymer, L. J. (2001). Quantification of *Stachybotrys chartarum* conidia in indoor dust using real time, fluorescent probe-based detection of PCR products. *J. Exposure Anal. Environ. Epidemiol.* **11**, 12−20.

Rohr, U.-P., Wulf, M.-A., Stahn, S., Steidl, U., Haas, R. and Kronenwett, R. (2002). Fast and reliable titration of recombinant adeno-associated virus type-2 using quantitative real-time PCR. *J. Virol. Meth.* **106**, 81−88.

Rollag, H., Sagedal, S., Holter, E., Degre, M., Ariansen, S. and Nordal, K. P. (1998). Diagnosis of cytomegalovirus infection in kidney transplant recipients by a quantitative RNA-DNA hybrid capture assay for cytomegalovirus DNA in leukocytes. *Eur. J. Clin. Microbiol. Infect. Dis.* **17**, 124−127.

Rosenstraus, M., Wang, Z., Chang, S.-Y., DeBonville, D. and Spadoro, J. P. (1998). An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. *J. Clin. Microbiol.* **36**, 191–197.

Ruan, Y. J., Wei, C. L., Ee, A. L., Vega, V. B., Thoreau, H., Su, S. T., Chia, J. M., Ng, P., Chiu, K. P., Lim, L., Zhang, T., Peng, C. K., Lin, E. O., Lee, N. M., Yee, S. L., Ng, L. F., Chee, R. E., Stanton, L. W., Long, P. M. and Liu, E. T. (2003). Comparative full-length genome sequence analysis of 14 SARS coronavirus isolates and common mutations associated with putative origins of infection. *Lancet* **361**, 1779–1785.

Rudi, K., Nogva, H. K., Moen, B., Nissen, H., Bredholt, S., Møretrø, T., Naterstad, K. and Holck, A. (2002). Development and application of new nucleic acid-based technologies for microbial community analyses in food. *Int. J. Food Microbiol.* **78**, 171–180.

Rutledge, R. J. and Côte, C. (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Res.* **31**, e93.

Ryncarz, A. J., Goddard, J., Wald, A., Huang, M.-L., Roizman, B. and Corey, L. (1999). Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. *J. Clin. Microbiol.* **37**, 1941–1947.

Sanburn, N. and Cornetta, K. (1999). Rapid titer determination using quantitative real-time PCR. *Gene Ther.* **6**, 1340–1345.

Schaade, L., Kockelkorn, P., Ritter, K. and Kleines, M. (2000). Detection of cytomegalovirus DNA in human specimens by LightCycler PCR. *J. Clin. Microbiol.* **38**, 4006–4009.

Schalasta, G., Arents, A., Schmid, M., Braun, R. W. and Enders, G. (2000a). Fast and type-specific analysis of herpes simplex virus types 1 and 2 by rapid PCR and fluorescence melting-curve-analysis. *Infection* **28**, 85–91.

Schalasta, G., Eggers, M., Schmid, M. and Enders, G. (2000b). Analysis of human cytomegalovirus DNA in urines of newborns and infants by means of a new ultrarapid real-time PCR-system. *J. Clin. Virol.* **19**, 175–185.

Scherr, M., Battmer, K., Blömer, U., Ganser, A. and Grez, M. (2001). Quantitative determination of lentiviral vector particle numbers by real-time PCR. *Biotechniques* **31**, 520–526.

Schoder, D., Schmalwieser, A., Schauberger, G., Kuhn, M., Hoorfar, J. and Wagner, M. (2003). Physical characteristics of six new thermocyclers. *Clin. Chem.* **49**, 960–963.

Schutten, M., van den Hoogen, B., van der Ende, M. E., Gruters, R. A., Osterhaus, A. D. M. E. and Niesters, H. G. M. (2000). Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. *J. Virol. Meth.* **88**, 81–87.

Schütz, E., von Ashen, N. and Oellerich, M. (2000). Genotyping of eight thiopurine methyltransferase mutations: three-color multiplexing, two-color/shared anchor, and fluorescence-quenching hybridization probe assays based on thermodynamic nearest-neighbour probe design. *Clin. Chem.* **46**, 1728–1737.

Selvey, S., Thompson, E. W., Matthaei, K., Lea, R. A., Irving, M. G. and Griffiths, L. R. (2001). β-actin – an unsuitable internal control for RT-PCR. *Mol. Cell. Probes* **15**, 307–311.

Selvin, P. (1995). Fluorescence resonance energy transfer. *Meth. Enzymol.* **246**, 300–334.

Semighini, C. P., Marins, M., Goldman, M. H. S. and Goldman, G. H. (2002). Quantitative analysis of the relative transcript levels of ABC transporter Atr genes in *Aspergillus nidulans* by real-time reverse transcription-PCR assay. *Appl. Environ. Microbiol.* **68**, 1351–1357.

Sharp, J. G. and Chan, W. C. (1999). Detection and relevance of minimal disease in lymphomas. *Cancer Metastasis Rev.* **18**, 127–142.

Shaw, R. D., Hempson, S. J. and Mackow, E. R. (1995). Rotavirus diarrhea is caused by nonreplicating viral particles. *J. Virol.* **69**, 5946–5950, Erratum: *J. Virol.* 1996. **70** (8), 5740.

Shrestha, N. K., Tuohy, M. J., Hall, G. S., Isada, C. M. and Procop, G. W. (2002). Rapid identification of *Staphylococcus aureus* and the mecA gene from BacT/ALERT blood culture bottles by using the LightCycler system. *J. Clin. Microbiol.* **40**, 2659–2661.

Siebert, P. D. and Larrick, J. W. (1992). Competitive PCR. *Nature* **359**, 557–558.

Simeonov, A. and Nikiforov, T. T. (2002). Single nucleotide polymorphism genotyping using short, fluorescently labeled locked nucleic acid (LNA) probes and fluorescence polarization detection. *Nucleic Acids Res.* **30**, e91.

Singh, S. K., Nielsen, P., Koshkin, A. A. and Wengel, J. (1998). LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. *Chem. Commun.* 455–456.

Sintchenko, V., Iredell, J. R. and Gilbert, G. L. (1999). Is it time to replace the petri dish with PCR? Application of culture-independent nucleic acid amplification in diagnostic bacteriology: expectations and reality. *Pathology* **31**, 436–439.

Smith, I. L., Halpin, K., Warrilow, D. and Smith, G. A. (2001). Development of a fluorogenic RT-PCR assay (TaqMan) for the detection of Hendra virus. *J. Virol. Meth.* **98**, 33–40.

Smith, I. L., Northill, J. A., Harrower, B. J. and Smith, G. A. (2002). Detection of Australian bat lyssavirus using a fluorogenic probe. *J. Clin. Virol.* **25**, 285–291.

Solinas, A., Brown, L. J., McKeen, C., Mellor, J. M., Nicol, J. T. G., Thelwell, N. and Brown, T. (2001). Duplex scorpion primers in SNP analysis and FRET applications. *Nucleic Acids Res.* **29**, e96.

Song, E. S., Lee, V., Surh, C. D., Lynn, A., Brumm, D., Jolly, D. J., Warner, J. F. and Chada, S. (1997). Antigen presentation in retroviral vector-mediated gene transfer in vivo. *Proc. Natl Acad. Sci. USA* **94**, 1943–1948.

Song, J., Ohkura, T., Sugimoto, M., Mori, Y., Inagi, R., Yamanishi, K., Yoshizaki, K. and Nishimoto, N. (2002). Human interleukin-6 induces human herpesvirus-8 replication in a body cavity-based lymphoma cell line. *J. Med. Virol.* **68**, 404–411.

Stender, H., Fiandaca, M., Hyldig-Nielsen, J. J. and Coull, J. (2002). PNA for rapid microbiology. *J. Microbiol. Meth.* **48**, 1–17.

Stephenson, J. (2003). Monkeypox outbreak a reminder of emerging infections vulnerabilities. *JAMA* **290**, 23–24.

Stevens, S. J. C., Verkuijlen, S. A. W. M., van den Brule, A. J. C. and Middeldorp, J. M. (2002). Comparison of quantitative competitive PCR with LightCycler-based PCR for measuring Epstein-Barr virus DNA load in clinical specimens. *J. Clin. Microbiol.* **40**, 3986–3992.

Stöcher, M., Leb, V., Hölzl, G. and Berg, J. (2002). A simple approach to the generation of heterologous competitive internal controls for real-time PCR assays on the LightCycler. *J. Clin. Virol.* **25**, S47–S53.

Stocher, M., Leb, V., Bozic, M., Kessler, H. H., Halwachs-Baumann, G., Landt, O., Stekel, H. and Berg, J. (2003). Parallel detection of five human herpes virus DNAs by a set of real-time polymerase chain reactions in a single run. *J. Clin. Virol.* **26**, 85–93.

Stockton, J., Ellis, J. S., Saville, M., Clewley, J. P. and Zambon, M. C. (1998). Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *J. Clin. Microbiol.* **36**, 2990–2995.

Stordeur, P., Poulin, L. F., Craciun, L., Zhou, L., Schandené, L., de Lavareille, A., Goriely, S. and Goldman, M. (2002). Cytokine mRNA quantification by real-time PCR. *J. Immunol. Meth.* **259**, 55–64.

Stram, Y., Kuznetzova, L., Guini, M., Rogel, A., Meirom, R., Chai, D., Yadin, H. and Brenner, J. (2004). Detection and quantitation of Akabane and Aino viruses by multiplex real-time reverse-transcriptase PCR. *J. Virol. Meth.* **116**, 147–154.

Strieter, R. M., Belperio, J. A. and Keane, M. P. (2003). Host innate defenses in the lung: the role of cytokines. *Curr. Opin. Infect. Dis.* **16**, 193–198.

Stryer, L. and Haugland, R. P. (1967). Energy transfer: a spectroscopic ruler. *Proc. Natl Acad. Sci. USA* **58**, 719–726.

Suzuki, K., Aoki, K., Ohnami, S., Yoshida, K., Kazui, T., Kato, N., Inoue, K., Kohara, M. and Yoshida, T. (2003). Adenovirus-mediated gene transfer of interferon α inhibits hepatitis C virus replication in hepatocytes. *Biochem. Biophys. Res. Commun.* **307**, 814–819.

Svanvik, N., Sehlstedt, U., Sjöback, R. and Kubista, M. (2000). Detection of PCR products in real time using light-up probes. *Anal. Biochem.* **287**, 179–182.

Svanvik, N., Westman, G., Wang, D. and Kubista, M. (2001). Light-up probes: thiazole orange-conjugated peptide nucleic acid for the detection of target nucleic acid in homogeneous solution. *Anal. Biochem.* **281**, 26–35.

Swan, D. C., Tucker, R. A., Holloway, B. P. and Icenogle, J. P. (1997). A sensitive, type-specific, fluorogenic probe assay for detection of human papillomavirus DNA. *J. Clin. Microbiol.* **35**, 886–891.

Sykes, P. J., Brisco, M. J., Snell, L. E., Dolman, G., Neoh, S.-H., Peng, L.-M. and Toogood, I. (1998). Minimal residual disease in childhood acute lymphoblastic leukaemia quantified by aspirate and trephine: is the disease multifocal? *Br. J. Haematol.* **103**, 60–65.

Takaya, K., Ogawa, Y., Hiraoka, J., Hosoda, K., Yamori, Y. and Nakao, K. (1996). Nonesense mutation of leptin receptor in the obese spontaneously hypertensive Koletsky rat. *Nat. Gen.* **14**, 130–131.

Tan, T. Y., Corden, S., Barnes, R. and Cookson, B. (2001). Rapid identification of methicillin-resistant *Staphylococcus aureus* from positive blood cultures by real-time fluorescence PCR. *J. Clin. Microbiol.* **39**, 4529–4531.

Tanaka, N., Kimura, H., Hoshino, Y., Kato, K., Yoshikawa, T., Asano, Y., Horibe, K., Kojima, S. and Morishima, T. (2000a). Monitoring four herpesviruses in unrelated cord blood transplantation. *Bone Marrow Transpl.* **26**, 1193–1197.

Tanaka, N., Kimura, H., Iida, K., Saito, Y., Tsuge, I., Yoshimi, A., Matsuyama, T. and Morishima, T. (2000b). Quantitative analysis of cytomegalovirus load using a real-time PCR assay. *J. Med. Virol.* **60**, 455–462.

Tanriverdi, S., Tanyeli, A., Baslamisli, F., Köksal, F., Kilinç, Y., Feng, X., Batzer, G., Tzipori, S. and Widmer, G. (2002). Detection and genotyping of oocysts of *Cryptosporidium parvum* by real-time PCR and melting curve analysis. *J. Clin. Microbiol.* **40**, 3237–3244.

Tarnuzzer, R. W., Macauley, S. P., Farmerie, W. G., Caballero, S., Ghassemifar, M. R., Anderson, J. T., Robinson, C. P., Grant, M. B., Humphreys-Beher, M. G., Franzen, L., Peck, A. B. and Schultz, G. S. (1996). Competitive RNA templates for detection and quantitation of growth factors, cytokines, extracellular matrix components and matrix metalloproteinases by RT-PCR. *Biotechniques* **20**, 670–674.

Tasker, S., Helps, C. R., Day, M. J., Gruffydd-Jones, T. J. and Harbour, D. A. (2003). Use of real-time PCR to detect and quantify *Mycoplasma haemofelis* and *Candidatus Mycoplasma haemominutum* DNA. *J. Clin. Microbiol.* **41**, 439–441.

Tasker, S., Helps, C. R., Day, M. J., Harbour, D. A., Gruffydd-Jones, T. and Lappin, M. R. (2004). Use of Taqman PCR to determine the response of *Mycoplasma haemofelis* infection to antibiotic treatment. *J. Microbiol. Meth.* **56**, 63–71.

Taswell, C. (1981). Limiting dilution assays for the determination of immuno-competent cell frequencies. *J. Immunol.* **126**, 1614–1619.

Taylor, M. J., Hughes, M. S., Skuce, R. A. and Neill, S. D. (2001). Detection of *Mycobacterium bovis* in bovine clinical specimens using real-time fluorescence and fluorescence resonance energy transfer probe rapid-cycle PCR. *J. Clin. Microbiol.* **39**, 1272–1278.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. and Heinen, E. (1999). Housekeeping genes as internal standards: use and limits. *J. Biotech.* **75**, 291–295.

Thelwell, N., Millington, S., Solinas, A., Booth, J. and Brown, T. (2000). Mode of action and applictation of Scorpion primers to mutation detection. *Nucleic Acids Res.* **28**, 3752–3761.

Todd, A. V., Fuery, C. J., Impey, H. L., Applegate, T. L. and Haughton, M. A. (2000). DzyNA-PCR: use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format. *Clin. Chem.* **46**, 625–630.

Torres, M. J., Criado, A., Palomares, J. C. and Aznar, J. (2000). Use of real-time PCR and fluorimetry for rapid detection of rifampin and isoniazid resistance-associated mutations in *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **38**, 3194–3199.

Tsang, K. W., Ho, P. L., Ooi, G. C., Yee, W. K., Wang, T., Chan-Yeung, M., Lam, W. K., Seto, W. H., Yam, L. Y., Cheung, T. M., Wong, P. C., Lam, B., Ip, M. S., Chan, J., Yuen, K. Y. and Lai, K. N. (2003). A cluster of cases of severe acute respiratory syndrome in Hong Kong. *N. Engl. J. Med.* **348**, 1977–1985.

Tseng, S. Y., Macool, D., Elliott, V., Tice, G., Jackson, R., Barbour, M. and Amorese, D. (1997). An homogeneous fluorescence polymerase chain reaction assay to identify *Salmonella*. *Anal. Biochem.* **245**, 207–212.

Tuma, R. S., Beaudet, M. P., Jin, X., Jones, L. J., Cheung, C.-Y., Yue, S. and Singer, V. L. (1999). Characterization of SYBR gold nucleic acid gel stain: a dye optimised for use with 300-nm ultraviolet transilluminators. *Anal. Biochem.* **268**, 278–288.

Tyagi, S. and Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* **14**, 303–308.

Tyagi, S., Bratu, D. P. and Kramer, F. R. (1998). Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.* **16**, 49–53.

Tyagi, S., Marras, S. A. E. and Kramer, F. R. (2000). Wavelength-shifting molecular beacons. *Nat. Biotechnol.* **18**, 1191–1196.

Uhl, J. R., Bell, C. A., Sloan, L. M., Espy, M. J., Smith, T. F., Rosenblatt, J. E. and Cockerill, F. R. III (2002). Application of rapid-cycle real-time polymerase chain reaction for the detection of microbial pathogens: the Mayo-Roche rapid anthrax test. *Mayo Clin. Proc.* **77**, 673–680.

Vaisse, C., Halaas, J. L., Horvath, C. M., Darnell, J. E., Stoffel, M. and Friedman, J. M. (1996). Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat. Gen.* **14**, 95–97.

Valentine-Thon, E. (2002). Quality control in nucleic acid testing-where do we stand? *J. Clin. Virol.* **25**, S13–S21.

van der Vliet, G. M. E., Hermans, C. J. and Klatser, P. R. (1993). Simple colorimetric microtiter plate hybridization assay for detection of amplified *Mycobacterium leprae* DNA. *J. Clin. Microbiol.* **31**, 665–670.

van Elden, L. J. R., Nijhuis, M., Schipper, P., Schuurman, R. and van Loon, A. M. (2001). Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *J. Clin. Microbiol.* **39**, 196–200.

van Santen, V. L., Kaltenboeck, B., Joiner, K. S., Macklin, K. S. and Norton, R. A. (2004). Real-time quantitative PCR-based serum neutralization test for detection

and titration of neutralizing antibodies to chicken anemia virus. *J. Virol. Meth.* **115**, 123–135.

Versalovic, J. and Lupski, J. R. (2002). Molecular detection and genotyping of pathogens: more accurate and rapid answers. *Trends Microbiol.* **10**, S15–S21.

Verstrepen, W. A., Kuhn, S., Kockx, M. M. and van de Vyvere, M. E. (2001). Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. *J. Clin. Microbiol.* **39**, 4093–4096.

Verstrepen, W. A., Bruynseels, P. and Mertens, A. H. (2002). Evaluation of a rapid real-time RT-PCR assay for the detection of enterovirus RNA in cerebrospinal fluid specimens. *J. Clin. Virol.* **25**, S39–S43.

Vet, J. A. M., Majithia, A. R., Marras, S. A. E., Tyagi, S., Dube, S., Poiesz, B. J. and Kramer, F. R. (1999). Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl Acad. Sci. USA* **96**, 6394–6399.

Vilcek, Š. and Paton, D. J. (2000). A RT-PCR assay for the rapid recognition of border disease virus. *Vet. Res.* **31**, 437–445.

Walker, G. T., Little, M. C., Nadeau, J. G. and Shank, D. D. (1992). Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc. Natl Acad. Sci. USA* **89**, 392–396.

Walker, R. A., Saunders, N., Lawson, A. J., Lindsay, E. A., Dassama, M., Ward, L. R., Woodward, M. J., Davies, R. H., Liebana, E. and Threlfall, E. J. (2001). Use of a LightCycler gyrA mutation assay for rapid identification of mutations conferring decreased susceptibility to ciprofloxacin in multiresistant *Salmonella enterica* serotype typhimurium DT104 isolates. *J. Clin. Microbiol.* **39**, 1443–1448.

Wall, S. J. and Edwards, D. R. (2002). Quantitative reverse transcription-polymerase chain reaction (RT-PCR): a comparison of primer-dropping, competitive, and real-time RT-PCRs. *Anal. Biochem.* **300**, 269–273.

Wang, T. and Brown, M. J. (1999). mRNA quantitation by real-time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal. Biochem.* **269**, 198–201.

Wang, Z. and Spadoro, J. (1998).Determination of target copy number of quantitative standards used in PCR based diagnostic assays In *Gene Quantification* (F. Ferré, ed.). Birkhauser, Boston.

Watkins-Reidel, T., Woegerbauer, M., Hollemann, D. and Hufnagl, P. (2002). Rapid diagnosis of enterovirus infections by real-time PCR on the LightCycler using the TaqMan format. *Diagn. Microbiol. Infect. Dis.* **42**, 99–105.

Watzinger, F., Hörth, E. and Lion, T. (2001). Quantitation of mRNA expression by competitive PCR using non-homologous competitors containing a shifted restriction site. *Nucleic Acids Res.* **29**, e52.

Weigl, J. A. I., Puppe, W., Gröndahl, B. and Schmitt, H.-J. (2000). Epidemiological investigation of nine respiratory pathogens in hospitalized children in Germany using multiplex reverse-transcriptase polymerase chain reaction. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**, 336–343.

Weinberger, K. M., Wiedenmann, E., Böhm, S. and Jilg, W. (2000). Sensitive and accurate detection of hepatitis B virus DNA using a kinetic fluorescence detection system (TaqMan PCR). *J. Virol. Meth.* **85**, 75–82.

Weis, J. H., Tan, S. S., Martin, B. K. and Wittwer, C. T. (1992). Detection of rare mRNAs via quantitative RT-PCR. *TIG* **8**, 263–264.

Wellinghausen, N., Frost, C. and Marre, E. (2001). Detection of Legionellae in hospital water samples by quantitative real-time LightCycler PCR. *Appl. Environ. Microbiol.* **67**, 3985–3993.

Wetmur, J. G. (1991). DNA probes: applications of the principles of nucleic acid hybridization. *Crit. Rev. Biochem. Mol. Biol.* **26**, 227–259.

Whelan, A. C. and Persing, D. H. (1996). The role of nucleic acid amplification and detection in the clinical microbiology laboratory. *Annu. Rev. Gen.* **50**, 349−373.

Whiley, D. M., Mackay, I. M. and Sloots, T. P. (2001). Detection and differentiation of human polyomaviruses JC and BK by LightCycler PCR. *J. Clin. Microbiol.* **39**, 4357−4361.

Whiley, D. M., LeCornec, G. M., Mackay, I. M., Siebert, D. J. and Sloots, T. P. (2002a). A real-time PCR assay for the detection of *Neisseria gonorrhoeae* by LightCycler. *Diagn. Microbiol. Infect. Dis.* **42**, 85−89.

Whiley, D. M., Syrmis, M. W., Mackay, I. M. and Sloots, T. P. (2002b). Detection of human respiratory syncytial virus in respiratory samples by LightCycler reverse transcriptase PCR. *J. Clin. Microbiol.* **40**, 4418−4422.

Whiley, D. M., Crisante, M. E., Syrmis, M. W., Mackay, I. M. and Sloots, T. P. (2003a). Detection of *Neisseria meningitidis* by LightCycler PCR. *Pathology* **35**, 347−349.

Whiley, D. M., Syrmis, M. W., Mackay, I. M. and Sloots, T. P. (2003b). Preliminary Comparison of Three LightCycler PCR assays for the detection of herpes simplex virus in swab specimens. *Eur. J. Clin. Microbiol. Infect. Dis.* **22**, 764−767.

Whitcombe, D., Theaker, J., Guy, S. P., Brown, T. and Little, S. (1999). Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* **17**, 804−807.

White, I. and Campbell, T. C. (2000). Quantitation of cell-free and cell-associated Kaposi's sarcoma associated herpesvirus DNA by real-time PCR. *J. Clin. Microbiol.* **38**, 1992−1995.

White, P. A., Pan, Y., Freeman, A. J., Marinos, G., Ffrench, R. A., Lloyd, A. R. and Rawlinson, W. D. (2002). Quantification of hepatitis C virus in human liver and serum samples by using LightCycler reverse transcriptase PCR. *J. Clin. Microbiol.* **40**, 4346−4348.

Whitley, R. J. (2003). Smallpox: a potential agent of bioterrorism. *Antiviral Res.* **57**, 7−12.

WHO, (1980). Declaration of global eradication of smallpox. *Wkly Epidemiol. Rec.* **55**, 145−152.

WHO, (2003). Acute respiratory syndrome. China, Hong Kong Special Administrative Region of China, and Vietnam. *Wkly Epidemiol. Rec.* **78**, 73−74.

Wilhelm, J., Hahn, M. and Pingoud, A. (2001a). Influence of DNA target melting behavior on real-time PCR quantification. *Clin. Chem.* **46**, 1738−1743.

Wilhelm, J., Pingoud, A. and Hahn, M. (2001b). Comparison between Taq DNA polymerase and its Stoffel fragment for quantitative real-time PCR with hybridization probes. *Biotechniques* **30**, 1052−1062.

Witney, A. A., Doolan, D. L., Anthony, R. M., Weiss, W. R., Hoffman, S. L. and Carucci, D. J. (2001). Determining liver stage parasite burden by real time quantitative PCR as a method for evaluating pre-erythrocytic malaria vaccine efficacy. *Mol. Biochem. Parasitol.* **118**, 233−245.

Wittwer, C. T., Fillmore, G. C. and Garling, D. J. (1990). Minimizing the time required for DNA amplification by efficient heat transfer to small samples. *Anal. Biochem.* **186**, 328−331.

Wittwer, C. T., Herrmann, M. G., Moss, A. A. and Rasmussen, R. P. (1997a). Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* **22**, 130−138.

Wittwer, C. T., Ririe, K. M., Andrew, R. V., David, D. A., Gundry, R. A. and Balis, U. J. (1997b). The LightCycler™: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176−181.

Wolffs, P., Grage, H., Hagberg, O. and Rådstrom, P. (2004). Impact of DNA polymerases and their buffer systems on quantitative real-time PCR. *J. Clin. Microbiol.* **42**, 408−411.

Real-time Fluorescent PCR Techniques to Study

Wolk, D. M., Schneider, S. K., Wengenack, N. L., Sloan, L. M. and Rosenblatt, J. E. (2002). Real-time PCR method for detection of *Encephalitozoon intestinalis* from stool specimens. *J. Clin. Microbiol.* **40**, 3922–3928.

Woo, T. H. S., Patel, B. K. C., Smythe, L. D., Symonds, M. L., Norris, M. A. and Dohnt, M. F. (1997). Identification of pathogenic *Leptospira* genospecies by continuous monitoring of fluorogenic hybridization probes during rapid-cycle PCR. *J. Clin. Microbiol.* **35**, 3140–3146.

Wood, L. (2003). Questions about comparative genomics of SARS coronavirus isolates. *Lancet* **362**, 578–579.

Woodford, N., Tysall, L., Auckland, C., Stockdale, M. W., Lawson, A. J., Walker, R. A. and Livermore, D. M. (2002). Detection of oxazolidinone-resistant *Enterococcus faecalis* and *Enterococcus faecium* strains by real-time PCR and PCR-restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **40**, 4298–4300.

Yin, J. L., Shackel, N. A., Zekry, A., McGuinness, P. H., Richards, C., van der Putten, K., McCaughan, G. W., Eris, J. M. and Bishop, G. A. (2001). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR green I. *Immunol. Cell. Biol.* **79**, 213–221.

Zerr, D. M., Huang, M.-L., Corey, L., Erickson, M., Parker, H. L. and Frenkel, L. M. (2000). Sensitive method for detection of human herpesvirus 6 and 7 in saliva collected in field studies. *J. Clin. Microbiol.* **38**, 1981–1983.

Zhai, J., Briese, T., Dai, E., Wang, X., Pang, X., Du, Z., Liu, H., Wang, J., Wang, H., Guo, Z., Chen, Z., Jiang, L., Zhou, D., Han, Y., Jabado, O., Palacios, G., Lipkin, W. I. and Yang, R. (2004). Real-time polymerase chain reaction for detecting SARS coronavirus, Beijing, 2003. *Emerg. Infect. Dis.* **10**, 300–303.

Zhang, J., Yankaskas, J. R. and Engelhardt, J. F. (1996). In vivo analysis of fluid transport in cystic fibrosis airway epithelia of bronchial xenografts. *Am. J. Physiol.* **270**, C1326–C1335.

Zhang, Y., Zhang, D., Li, W., Chen, J., Peng, Y. and Cao, W. (2003). A novel real-time quantitative PCR method using attached universal template probe. *Nucleic Acids Res.* **31**, e123.

Zimmermann, K. and Manhalter, J. W. (1996). Technical aspects of quantitative competitive PCR. *Biotechniques* **21**, 268–279.