CHAPTER 7

Quantitative Real-time PCR Analysis

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7.1 Introduction

The sensitivity of analysis achievable with PCR has led to the technology being adopted across a range of sectors. For many applications a quantitative result is required, which has driven the development of a range of strategies to determine the amount of starting material in a sample. Approaches such as competitive PCR[1] and limiting dilution analysis[2] have been used as routes to quantification, although the variable nature of the PCR process and the amplification of the target to a maximal level irrespective of the starting amount of target limit the accuracy of these methods.[3]

The advent of kinetic or real-time PCR[4] has overcome many of the limitations of earlier strategies, by monitoring the increase in product generated during the course of the reaction, in 'real time'. Quantitative approaches are based on the time or cycle at which amplification is first detected, rather than requiring quantification of PCR products, and the principle is illustrated schematically in Figure 7.1. A range of samples of known target content are usually amplified together with the samples under test, and the accumulation of PCR product in each cycle is determined. Alternatively the signal from two targets may be compared to determine a relative measure of quantification, and this is often used in measurement of gene expression which is considered in more detail in Chapter 9.

Here a fluorescent reporter assay is used to monitor increase in fluorescence at each PCR cycle. The point at which the signal becomes detectable, or crosses some arbitrary threshold value, is determined for each standard and sample. These values are then plotted against the amount of target in the standards to produce a calibration curve, and the amount of target in the unknown samples can then be interpolated from the graph.[5]

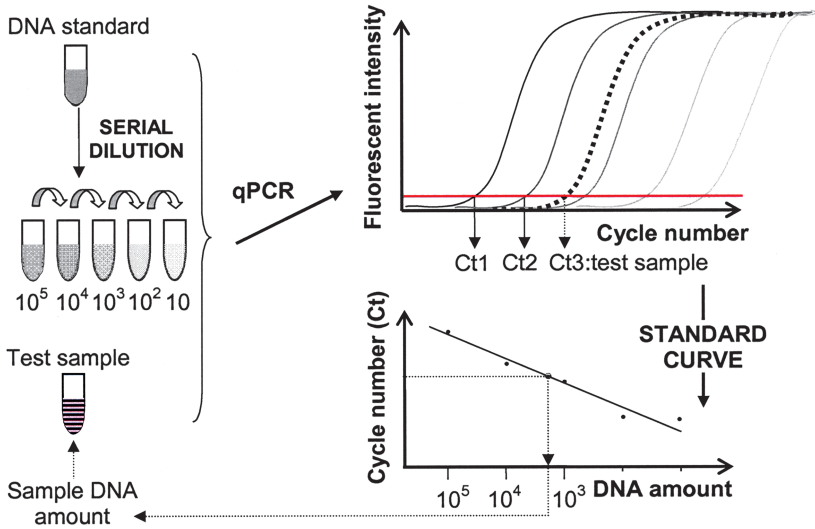

Figure 7.1 Schematic representation of the basis of real-time PCR using a quantitative standard.

The linear relationship between the amount of starting material and the measured cycle threshold (Ct) values are maintained across several orders of magnitude, so assays based on quantitative PCR (qPCR) have an unusually large dynamic range. There are a number of other significant benefits in using real-time PCR analysis, including the greatly increased sensitivity associated with the use of fluorescent reporters and signal collection devices, and the rapid cycling times that are achievable on some instruments. In addition, homogeneous qPCR assays minimise the potential for cross-contamination compared with conventional methods as reaction vessels need not be opened in order to analyse amplification products, and also avoid variation introduced by gel analysis.

In short, real-time PCR offers the potential of well-characterised and highly sensitive quantitative analysis, although the diversity of instruments, detection chemistries, data handling methods and the lack of quantitative reference standards present significant challenges to measurement comparability.

7.2 Approaches to Product Detection

The key feature of quantitative real-time PCR is that the amount of product is measured at each cycle of the reaction, and thus requires simultaneous PCR amplification and product detection. The first assay of this type utilised simple incorporation of the fluorescent dye ethidium bromide into the PCR reaction, and the increase in signal resulting from the dye intercalating into the double-stranded PCR products was monitored using a CCD camera.[4] However, ethidium bromide signals will increase with increasing amounts of any

double-stranded DNA, and thus primer dimers and non-specific PCR products will all generate signals that are not distinguishable from a true positive reaction, therefore more specific reporter systems are preferable for many applications. In addition, more efficient fluorophores than ethidium bromide are available (Chapter 5), which have been successfully employed to improve the sensitivity of real-time assays. The majority of homogenous assays rely on the transfer of energy between fluorescent reporter and quencher molecules to generate specific signals. When oligonucleotide probes are excited the energy absorbed by the fluorophore may be emitted as fluorescence or may be transferred to a quencher and released as heat or light of a different wavelength. This energy transfer may occur through Förster Resonance Energy Transfer (FRET) if the emission and absorption spectra of the molecules overlap sufficiently or through non-FRET mechanisms by short-range contacts, which do not require any spectral overlap between the donor and acceptor molecules. Several of the most common approaches that have been developed to monitor PCR kinetics, and the labels used, will be described in the following sections.

7.2.1 The 5′ Nuclease Assay

The 5′ nuclease, or TaqMan™, assay[6] utilises FRET quenching to analyse PCR-amplified target DNA, although the original method was developed using a radioactive labelling approach.[7] The assay exploits the 5′-3′ nuclease activity of *Taq* DNA polymerase to cleave a dual-labelled oligonucleotide probe, labelled with fluorophore and quencher moieties on the 5′ and 3′ termini respectively. Little fluorescence is emitted from the intact probe due to efficient intramolecular quenching, as energy absorbed by the fluorophore is transferred to the quencher and dissipated as heat. However, during PCR amplification, TaqMan™ probes specifically hybridise to their target sequences and the 5′-3′ exonuclease activity of *Taq* polymerase cleaves the probes between fluorophore and quencher moieties. Enzymatic cleavage of TaqMan™ probes spatially separates fluorophore and quencher components, causing significant increase in fluorescence emission (Figure 7.2). With each cycle of denaturation, primer annealing and product extension, a molecule of reporter dye is liberated from a quencher moiety for each molecule of newly synthesised DNA. Therefore, the magnitude of the emission increase produced during amplification is

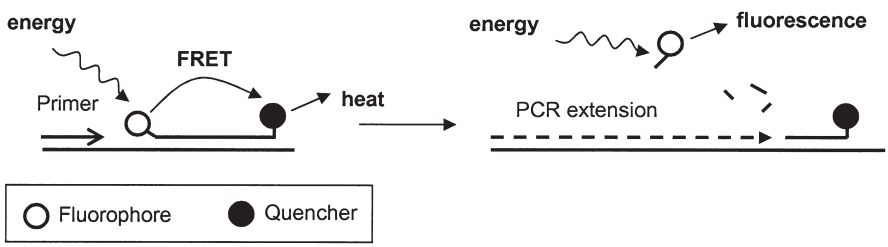

Figure 7.2 Illustration of the basis of the 5′ nuclease (TaqMan™) assay.

proportional to the amount of PCR product synthesised. Fluorescence emission may be monitored throughout the course of PCR, allowing the generation of a 'real-time' representation of target amplification. Polynucleotide targets differing by as little as a single nucleotide may be distinguished using TaqMan™ probes, since oligonucleotides hybridise to mismatched DNA targets with a significantly reduced efficiency. Careful design of TaqMan™ probes allows discrimination of polymorphic targets, where only perfectly matched probes are degraded during amplification generating increases in fluorescent signal.[6,8] TaqMan™ probes may be employed to detect and discriminate multiple targets in a single reaction, using oligonucleotides labelled with spectrally distinct fluorophores,[9] and a wide range of commercial assays have been developed. Specific software is available to facilitate the design of 5′ nuclease assays, and up-to-date guidelines can also be found on the Applied Biosystems website.[10] In brief, the melting temperature of the probe should be 10 °C higher than that of the primers, and should be located adjacent to one of the primers but not overlapping the primer binding site. The inclusion of a G at the 5′ end of the probe should be avoided, as this base may partially quench the signal from the fluorophore. As the requirement for a probe of much higher T_m than the primers can be a challenge in designing probes, molecules that are designed to bind to the minor groove of dsDNA can be employed (MGB probes) to achieve the required T_m without using lengthy sequences.[11] A similar improvement in probe binding and T_m can be achieved by using locked nucleic acid probes, LNAs,[12] which are RNA analogues with a structurally constrained sugar-phosphate backbone.

7.2.2 Molecular Beacons™

Molecular Beacons™ are essentially single-stranded oligonucleotide probes that are non-fluorescent in isolation, but become fluorescent upon hybridisation to target sequences.[13,14] Non-hybridised molecular beacons form stem-loop structures, possessing a fluorophore covalently linked to one end of the molecule and a quencher linked to the other, such that the hairpin of the beacon places the fluorophore moiety in close proximity with the quencher. Since the quencher component is commonly a non-fluorescent moiety, the energy it receives from the fluorophore is released as heat, such that fluorescence is not emitted from unhybridised probe. The loop portion of the molecular beacon molecule is a specific probe that is complementary to a nucleic acid sequence present in the target DNA. Probe-target duplexes are longer and more stable than the stem hybrids. Therefore, when molecular beacons hybridise to target sequences, they undergo a fluorogenic conformational change where fluorophore and quencher moieties become spatially separated, such that the fluorophore is no longer quenched and the molecular beacon fluoresces (Figure 7.3). In designing molecular beacons, a sequence between 10 and 40 nucleotides long is chosen in the centre of the PCR product, which is complementary to the target of interest. The sequence should be free of significant secondary structure, then the stem and loop structures are formed by adding 5–7 bases and a fluorophore

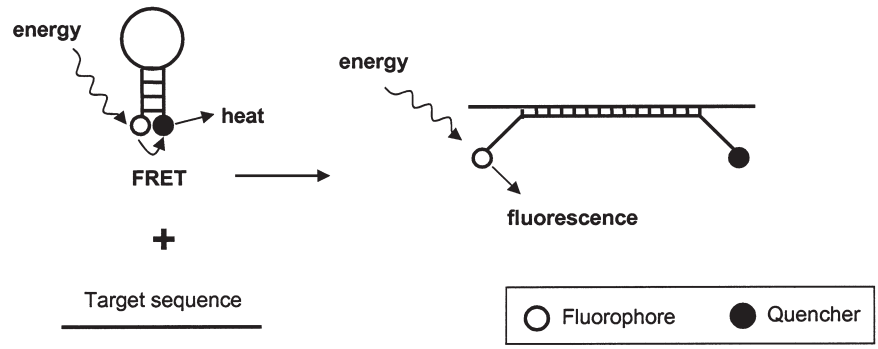

Figure 7.3 Target detection using Molecular Beacons™.

at the 5′ end and a complementary 5–7 nucleotides and a quencher at the other terminus.

The secondary structure of the molecular beacon conveys high specificity to the probe, allowing the identification of targets that differ by a single nucleotide, where only perfectly complementary probe-target duplexes are sufficiently stable to induce the fluorogenic conformation transition. Molecular Beacons™ may also be employed to detect and discriminate multiple targets in a single reaction, using probes that possess different fluorophores which emit light at spectrally distinct wavelengths[15] (Table 7.1).

Simpler linear probe systems have been developed, which only require a single labelled reporter. HyBeacons® have a single fluorescent label attached to an internal nucleotide, and show enhanced fluorescence on binding to a complementary target.[16] The ResonSense® system is based on FRET, but an intercalating fluorophore is used as the donor, and a single label acceptor is attached to either the 3′ or 5′ end of the probe or internally.[17] These simpler reporters are well suited to multiplexed assays.

7.2.3 Hybridisation Probes

Hybridisation probes are oligonucleotides that are singly labelled with a fluorophore. Two such oligonucleotides are required for each hybridisation probe assay, one labelled with a donor fluorophore and the other with an acceptor fluorophore.[18] Excitation of the donor fluorophore produces an emission spectrum that overlaps with the absorption spectrum of the acceptor fluorophore. Hybridisation probe pairs are designed to recognise adjacent nucleotide sequences within target molecules. In isolation, the acceptor oligonucleotide is not excited and does not generate a fluorescent signal. However, during hybridisation to the target sequences, the donor and acceptor probes are brought into close proximity, allowing fluorescence resonance energy transfer from the donor to the acceptor (Figure 7.4).

Fluorescent signal from the acceptor fluorophore is thus emitted when both probes are hybridised to the target molecule. When incorporated into PCR

Table 7.1 Primary λmax absorption and emission wavelengths of frequently used fluorophores, quenchers and fluorescent dyes.

Name	*Absorption max (nm)*	*Emission max (nm)*
	Fluorophores	
EDANS	336	490
FAM	492	515
Fluorescein	494	525
SYBR Green I	497	520
Ethidium bromide	518	605
JOE	520	548
TET	521	536
Yakima Yellow	525	548
VIC	528	546
HEX	535	556
Cy3	544	570
TAMRA	555	580
ROX	575	602
Texas Red	583	603
LC-RED 640	625	640
LC-RED 705	625	603
Cy5	647	667
	Quenchers	
DABCYL	471 (~400–550)	—
Deep Dark Quencher I	410 (~400–550)	—
Deep Dark Quencher II	630	
Eclipse	530	—
Black Hole Quencher-1	534 (~480–580)	—
Black Hole Quencher-2	579 (~559–650)	
Black Hole Quencher-3	672 (620–730)	
Iowa Black FQ	532	—
Iowa Black RQ	645	
QSY-7	571	—
QSY-21	660	

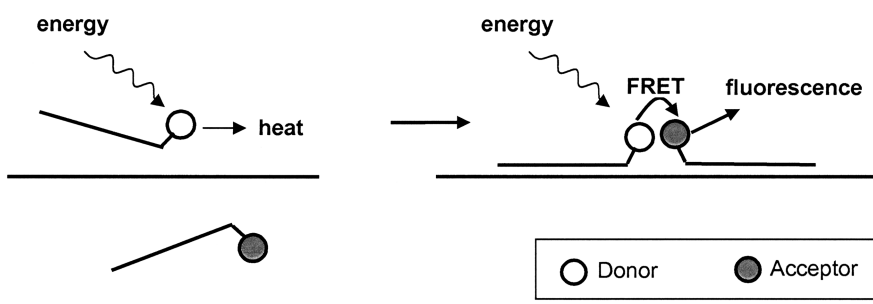

Figure 7.4 Target detection using hybridisation probes.

reactions, fluorescence from the acceptor probe is monitored once per cycle of amplification, allowing real-time measurement of product accumulation, where the amount of fluorescence emitted by the acceptor is proportional to the quantity of target synthesised. The use of several oligonucleotide probes bearing spectrally distinct acceptor fluorophores may be employed in a multiplexed analysis, to simultaneously detect and discriminate multiple targets in a single PCR reaction[19] (Table 7.1).

7.2.4 Scorpion™ Primers

In contrast to the systems already described, where the probe and PCR primers are located on separate DNA strands, Scorpion™ primers are designed so that the probe, primer and hence the amplified target are located on the same DNA molecule. Scorpion™ probes comprise a primer with an attached probe tail sequence, where the probe is contained within a stem-loop secondary structure similar to that of a molecular beacon.[20] In the unextended form, Scorpion™ primers are non-fluorescent due to fluorophore and quencher moieties being in close proximity. During PCR, the primer component of the Scorpion™ is extended at its 3′ end producing the homologous target sequence required for probe hybridisation. When the Scorpion™ probe sequence hybridises to the amplified target, the hairpin loop of the probe opens and the fluorophore and quencher moieties become spatially separated (Figure 7.5) causing significant increases in fluorescent emission. The fluorescent signal is produced concurrent with target amplification, allowing the amount of product to be monitored. A benefit of this system is that unimolecular binding events are kinetically favoured over bimolecular hybridisation.

7.2.5 Plexor™ Primer Technology

Plexor™ primer technology, available from Promega, is a relatively new approach that requires only two primers for sensitive and specific quantification of amplified DNA. The approach exploits the highly specific interaction between structurally modified G and C bases, isoguanine (iso-dG) and 5′-methylcytosine (iso-dC) respectively, which only base-pair with each other when incorporated into dsDNA.[21] The approach involves synthesising one of

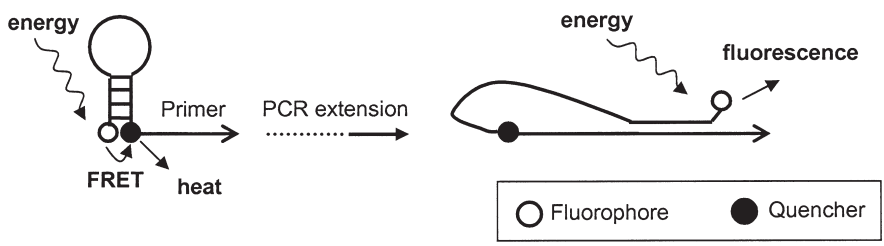

Figure 7.5 Target detection using a Scorpion™ probe.

the 2 PCR primers with an iso-dC base and a fluorophore at the 5′ end, and including iso-dG bases modified to include a quencher in the PCR reaction. As the amplification progresses, only modified iso-dG can be incorporated into products complementary to the primer, and thus the quencher is brought into close enough proximity to the fluorophore to effect quenching of the signal, at a level proportional to the amount of PCR product generated. An added benefit of this system is that the quenching is reversible, so melt curve analysis can be performed using this system.

7.2.6 Melting Curve Analysis

In addition to measuring the increase in product at each cycle, an analysis of the products generated in the reaction may be performed at the end of the amplification process. This is termed 'melt analysis' and is compatible with both intercalating dye reporter systems and those where the probe binds to the PCR product to achieve a change in fluorescence intensity. To perform the analysis the fluorescent signal is monitored as the temperature is gradually increased from around 50 to 95 °C, which results in an increase in fluorescence as the dsDNA or probe:product complex is dissociated. The change in fluorescence against temperature is usually plotted by instrument software, yielding peaks corresponding to the denaturation maxima of each double-stranded species present in the system (Figure 7.6). Primer-dimers, non-specific or mismatched sequences will generally have a lower melting temperature than the specific product of the reaction, and so can be distinguished by this post-PCR analysis.[22]

Recently, an extension of melt curve analysis has been developed, termed High Resolution Melt or HRM (Corbett Life Science), enabled by improvements in

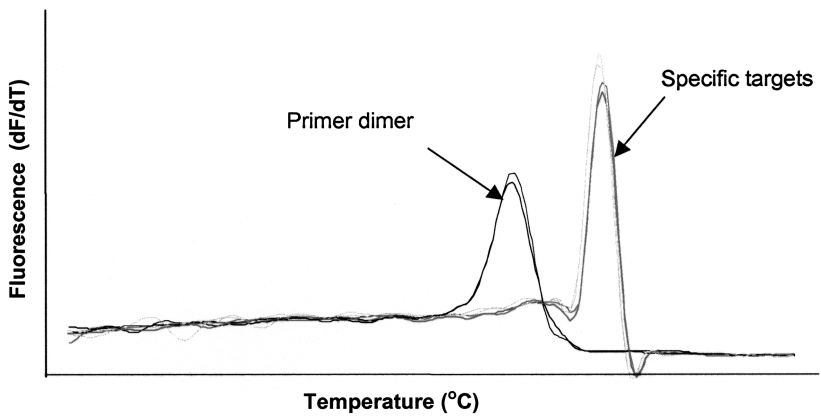

Figure 7.6 Schematic representation of a typical post-amplification melt analysis to differentiate specific PCR products from primer dimers. Melt analysis can also be used to identify wild type and mutant alleles, based on the lower melting temperature of unmatched species.

real-time instrument capabilities and the dyes used in analysis. Rapid data collection is required, with very high precision thermal resolution (down to 0.02 °C) and dedicated analysis software.

Samples are characterised based on very detailed measurement of their disassociation kinetics, and with more detailed melt profiles samples may be discriminated by length, GC content and sequence. Even single base-pair mismatches can be distinguished, allowing application of the method to detailed genotyping analysis, and thus this approach could potentially be exploited to replace the use of more complex probe reporter systems.[23]

7.2.7 Choice of Fluorophores

A range of dyes is available from various manufacturers, which may be used in constructing probes for use in the assays described here. Considerations in choosing a dye include the type of assay design, the limitation of the instrument in terms of excitation and signal capture wavelengths, and the overlap of fluorophore signals if multiplexing is required. The majority of fluorophores have wide emission spectra, and there is often a significant degree of overlap in signals (Figure 7.7). The labels used in a multiplexed assay must be chosen

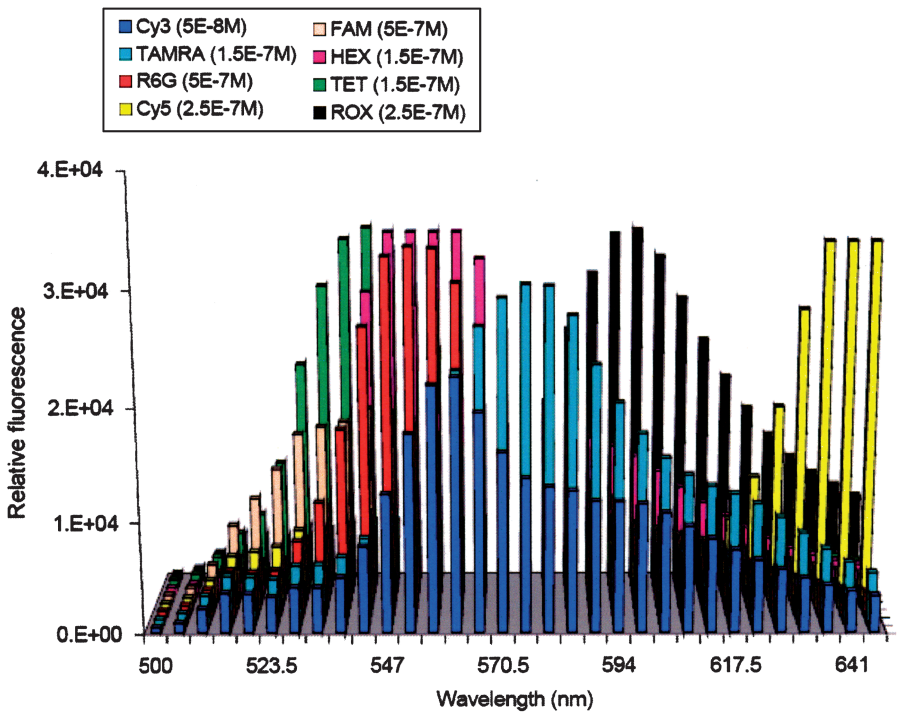

Figure 7.7 Spectra of a number of commonly used fluorophores measured using the ABI PRISM® 7700 SDS. The molar concentrations of each fluorophore are indicated in the key.

carefully to maximise spectral separation, and thus facilitate deconvolution of the multiplexed signals.

Usually assays are designed to utilise a universal donor molecule, which absorbs energy from the instrument light source and transfers it to a variety of adjacent reporter molecules. The signal from the reporters is distinguishable by the emission wavelengths, which are measured by the instrument. The ResonSense® assay is an example of this design, where the intercalating fluorophore is excited and transfers energy to a range of labelled probes. The alternative approach is to have a range of donor molecules which all may be excited by the light energy from the instrument, whilst a universal acceptor molecule is utilised as a quencher. The 'TaqMan™' assay run on the ABI real-time platforms is an example of the second type of assay, where TAMRA or an alternative quencher is paired with a variety of 5′ fluorophore labels.

In designing FRET-based assays there should be sufficient spectral overlap between the fluorophore-quencher pairs, such that the emission maximum of the donor is within the excitation range of the acceptor molecule. For example, fluorophores with an emission maximum between 500 and 550 nm, such as FAM and TET, are effectively quenched by dyes such as DABCYL (absorption maxima at 471 nm, with a range between 400 and 550 nm). By contrast, fluorophores with a higher emission maximum such as ROX, Texas Red and the Cy dyes are more effectively paired with quenchers with a higher absorption maximum (Table 7.1).

7.3 Range of Instruments

The number of real-time PCR instruments on the market is still increasing, offering a variety of options in terms of throughput, cycling times, flexibility and cost[10,24–30] (Table 7.2). The basic requirements of an instrument are to provide the temperature-controlled environment for PCR amplification, whilst providing light excitation and quantitative fluorescent signal collection of appropriate wavelength; thus both controlling and monitoring the amplification process. Instruments differ in many key features, including:

- Speed of reaction (heating and cooling rates);
- Precision and uniformity of the temperature control;
- Throughput/number of reactions performed at one time;
- Range of excitation wavelengths;
- Range of detection wavelengths;
- Sensitivity of detection device;
- Ease of use and capabilities of software;
- Flexibility of temperature profiles and chemistries within each run;
- Cost.

The choice of instrument will depend primarily on the range of applications for which it is required.

Table 7.2 Performance characteristics of a selection of commercially available real-time PCR instruments.

Instrument	*ABI PRISM® 7900HT SDS*	*ABI StepOne®*	*Stratagene Mx® 3000P® (4000) Systems*	*Corbett Rotor-Gene™ 6000*	*Roche Lightcycler® 480*	*Roche LightCycler® 2.0*	*Eppendorf MasterCycler®*	*Cepheid Smart Cycler®*	*BioGene InSyte*
Capacity	96 or 384	48-well block	96-well block	36 or 72 tubes	96 or 384	32 capillary	96-well block	16 to 96 tube	96 reactions
Reaction volume/μl	Standard: 25–100; Fast: 10–30; 384: 5–20	10–30	20–100, optimised for 25	5–100	96: 20–100; 384: 5–20	20 and 100	25–100	2–100	50
Reaction time (40 cycles)	Standard: 2 h; Fast: 33 min (96), 52 min (384)	40 mins/2 hours	2.5 hours (1.5 h)	30 minutes	40 min (384); 60 min (96)	30 min (30 cycles)	30 min	40 min	<20 min (35 cycles)
Excitation source and range	488 nm argon-ion laser	Single blue LED	Quartz Tungsten Halogen lamp 350 nm–750 nm	4 separate high-power LEDs; 470, 530, 585 and 625 nm	High-intensity xenon lamp 430–630 nm and 5 filters; 450, 483, 523, 558, 615 nm	Blue LED 470 nm	96 LEDs at 470 nm	4 channels: 450–495, 500–550, 565–590 and 630–650 nm	Solid-state blue laser 473 nm
Detector/ Emission range	Spectrograph and cooled CCD	Three emission filters, and photodiode	1 (4) scanning photomultiplier tubes (PMT),	PMT and 6 detection filters; 510, 555, 610,	CCD camera with 6 detection	6 channel photometer: 530, 560,	1- or 2-channel PMT; 520/550	4 channels; 510–527, 565–590,	32 channel spectrometer (520–720 nm)

	camera from 500–660 nm		with 4 filters; 350–830 nm	665, 570 and 610 nm	filters; 500, 533, 568, 610, 640 and 670 nm	610, 640, 670 and 705 nm	or 520/550/580/605 nm	606–650 and 670–750	
Temperature control	Peltier	Peltier	Peltier hybrid (resistive + convective)	Air heating and cooling, with centrifugation	Peltier with Therma-base heat distribution	Air heating and cooling	Normal and fast Impulse PCR blocks	Solid-state heater and forced air cooling	Electrically conducting polymer (ECP)
Temp. ramp rate °C/sec	Standard: ±1.6 °C/sec; Fast: ±3.0 °C/sec	Standard:±1.6 °C/sec; Fast:±2.2 °C/sec	Up to 2.5 °C/sec	10 °C/sec		20 °C/sec	Fast: 6 °C/sec heating, cooling 4.5 °C/sec	Heating, 10 °C/s; cooling 2.5 °C/s	Av. 15 °C/sec
Temp. uniformity	±0.50 °C	±0.50 °C	±0.25 °C	±0.01 °C	±0.1°C	±0.3 °C	35 °C ±0.3 °C, 90 °C±0.4 °C	±0.50 °C	0.01 °C resolution
Temp. range	4 °C–100 °C	4 °C–100 °C	25–95 °C	25–99 °C	40–95 °C	40–98 °C	4 °C–99 °C	50–99 °C	Ambient – 100 °C
W x D x H/cm	72 × 64 × 84	24.6 × 51 × 42.7	33 × 46 × 43	38 × 48 × 31.5	60 × 60 × 54.5	28 × 38.5 × 50.5	26 × 41 × 39.6	30 × 30 × 25	44 × 46 × 60
Weight/kg	82	23.6	20	17	55	22	24	10	45
Additional features	Auto loader (throughput 5000 wells/8h day)			Optical temperature validation system				Independently controlled reactions	

Instruments generally fall into two types; higher-throughput machines with 96 or 384 reaction capacity for processing batches of samples (for example the ABI PRISM® 7900HT and the Roche LC480) or more flexible instruments with faster reaction times and greater flexibility, such as the Cepheid SmartCycler®.

Instruments using a single wavelength excitation source are more limited in the variety of compatible fluorophores than those with a broader excitation range. For example the Eppendorf MasterCycler® utilises LED excitation at 470 nm, while Stratagene' Mx3000P® employs a halogen lamp with an excitation range from 350–750 nm. The fluorescence detector and the capability of the instrument for distinguishing different wavelength signals also influences the level of assay multiplexing that is achievable.

Uniform and precise temperature control is central to obtaining reproducible quantitative results, and thus there is a requirement for instruments to have effective thermal control systems. Many of the instruments on the market utilise Peltier-driven heating and cooling systems, which work by passing electric currents through semiconductor elements connected in series to effect temperature changes that are proportional to the currents applied. Exceptions are the Corbett Rotor-Gene™, which houses reaction tubes in a centrifugal rotor, and the Roche LightCycler® 2.0, which contains a rotor of glass capillaries, both relying on air heating and cooling in the reaction chamber to control sample temperature. High-speed centrifugation of samples in the Rotor-Gene™ ensures temperature homogeneity between samples, and air heating and cooling systems afford both instruments rapid reaction times. The latest versions of LightCycler® 2.0 software also enable the samples to be constantly rotated during amplification, thereby increasing thermal homogeneity. Newer electrically conducting polymer (ECP) heating technology is exploited in BioGene' InSyte real-time instrument, enabling precise individual tube thermal control and reduced run times. Miniaturisation has also seen the introduction of nanofluidic chips for qPCR analysis, which are biochips employing systems of integrated channels and valves to manipulate the reagents and house the amplification process.[31]

7.4 Practical Aspects of qPCR Analysis

A number of factors can affect the performance of qPCR, including the initial choice of target, probe and primer sequences, the concentration and type of reaction components, the thermal cycling conditions, the reaction vessels used and the preparation of the samples and any standards used. To ensure reliability of analytical results both the assay design and the reagents should be considered carefully.

7.4.1 Assay Design

A range of validated assays are available from a number of manufacturers, such as the TaqMan™ SNP Genotyping and Gene Expression Assays from Applied

Biosystems, and Invitrogen' D-LUX™ assays. The benefits of using pre-designed assays are that the reagents and methods are usually validated and quality-controlled, saving time and effort. However an appropriate assay may not be commercially available for many applications, and the cost of utilising off-the-shelf assays may be prohibitive. An online database of quantitative PCR primers and probes (QPPD) designed for human and mouse gene expression studies is also available on line.[32]

7.4.1.1 Target Sequence

In designing a new assay, choice of target sequence is typically the first consideration. The target ideally should not contain strong secondary structure as this can reduce the efficiency of oligonucleotide probe hybridisation, and this may be assessed using the Mfold[33] or similar structure prediction program, several of which are freely available online.[34] The target sequence should also be analysed for the existence of similar sequences that may interfere with the assay using a BLAST search[35] of sequence databases, and the assay region chosen to minimise any cross reactivity.

The amplicon size may affect assay efficiency and sensitivity; if too long the double-stranded PCR products may not denature efficiently at each cycle, and may preferentially re-hybridise in each cooling cycle before probe and primer sequences can bind. Typically amplicons of less than 150 bp are used, although amplicon size is not limited by these considerations in assays utilising fluorescent dyes or intercalating dyes.

7.4.1.2 Probe and Primer Design

Having chosen the target sequence, primers and probe sequences will be required, depending on the chosen assay format. Several manufacturers of real-time instrumentation and assay reagents provide software for this purpose, and a number of primer and reporter probe design packages are also freely accessible online, such as FastPCR[36] and AutoPrime.[37] For most applications, primers are designed to be fully complementary to template DNA sequences, and the basic considerations are similar to those for successful conventional PCR. Typically, primers should be designed to be 18–30 nucleotides in length to allow a reasonably high annealing temperature to be used during PCR. Primer pairs should be approximately the same length, should possess 40–60% GC content and should lack significant secondary structures or complementary regions. Regions of complementarity at the 3′ end of primers should be minimised to reduce the potential for the formation of primer dimer during amplification.

The design requirements for the individual types of fluorescent probes will not be discussed here, but can be found in the cited literature,[7,8,13,15,19,20] and generally the probe T_m should be higher than that of the primers to ensure that the probe has bound before the primers hybridise and extension begins. If the application requires discrimination of closely related target sequences, then

probes of between 15 and 30 nucleotides in length are recommended, as this gives a balance between forming sufficiently stable hybrids with target sequences, but retaining the sensitivity of melting temperatures to the presence of sequence mismatches. AT-rich target sequences may require probes that are greater than 30 nucleotides in length to form stable hybrids, and probes composed of peptide nucleic acid (PNA) or containing certain DNA base analogues (for example Propyne dC) may also be employed to form more stable interactions. Conversely, target sequences that are particularly GC rich may require probes that comprise fewer than 15 nucleotides for effective target discrimination, or alternatively DNA base analogues (such as N4 Ethyl dC) may be used to destabilise duplex interactions and lower the T_m of oligonucleotide probes.

The type of nucleotide mismatch that occurs within DNA duplexes strongly influences the stability of hybridisation. Mismatched interactions involving G, particularly G to T, are the least destabilising whilst interactions involving C are the most destabilising.[38] The position of the base mismatch, relative to the probe/target duplex, also influences the difference in stability between matched and mismatched interactions. Positions of mismatch located at duplex termini are significantly less destabilising than mismatches situated towards the centre of oligonucleotide probes,[39] thus allowing design of the probe position according to the specific application requirements.

7.4.2 PCR Master Mix

The qPCR reaction environment is usually provided by a master mix that includes buffer, dNTPs, thermostable polymerase and $MgCl_2$. Additional components such as ROX as a passive reference dye, and UNG with dUTP to prevent PCR product contamination, may also be included. Many instrument manufacturers provide reagents for use with particular instruments or assay/probe formats, although many reagents work well with a variety of assays and platforms. Use of commercial reagents affords benefits in terms of licence to perform qPCR, quality assurances and batch-to-batch consistency, although the cost is higher than reagents prepared in-house. Using complete systems from one manufacturer, from the assay design software through to the master mix and instrument settings, can reduce the number of factors requiring optimisation, thereby saving much time and effort.

7.4.2.1 Magnesium Chloride

The concentration of $MgCl_2$ is known to have an impact on both the specificity and the yield of PCR; insufficient Mg^{2+} results in poor yields as the polymerisation rate of *Taq* polymerase is low; however, if the level of Mg^{2+} is too high the specificity of the reaction is compromised. In contrast to conventional assays, homogenous assays require higher $MgCl_2$ concentrations of around 3–5 mM to achieve efficient target amplification and detection. The presence of $MgCl_2$ increases the rate of DNA hybridisation,[40] enabling efficient hybridisation during the rapid cycling conditions used in many instruments.

7.4.2.2 DNA Polymerase

The type of DNA polymerase employed in homogeneous PCR assays may affect the sensitivity and efficiency of target amplification, detection and discrimination. For example, certain enzymes such as *Z-Taq*™ (TaKaRa) exhibit higher processivities and rates of PCR extension than standard *Taq* polymerase. Increasing the speed of product synthesis may allow the reduction of PCR hold times and the overall duration of amplification, especially when combined with the fast temperature transition rates of rapid cycling instruments. GC rich target sequences may also require a high denaturation temperature, necessitating the use of polymerases such as Stoffel fragment and Deep Vent with increased thermal stability that support denaturation in excess of 95 °C.

The use of hot-start PCR may improve assay performance in applications where it is important to minimise the formation of primer-dimer and other non-specific PCR products, and has also been shown to improve the assays on the LightCycler®, possibly by reducing binding of the enzyme to the glass tube surfaces.[41]

The 5′-3′ exonuclease activity is vital for fluorescent signal generation and target detection in TaqMan™ style assays. Commercially available DNA polymerases have been demonstrated to generate variant amounts of fluorescent signal when employed in TaqMan™ assays performed on a LightCycler®[42], suggesting assay performance may be affected by the choice of enzyme. The majority of assays using TaqMan™ probes employ AmpliTaq Gold™ (ABI) for efficient 5′-3′ exonuclease activity and the large increases in fluorescence emission that it produces during amplification.

7.4.3 Cycling Conditions

The real-time platforms on the market are all supplied with detailed recommendations, and it is advisable to consult the manufacturer' literature for information on instrument operation and settings. A set of generalised assay conditions for homogenous assays using SYBR® Green on the LightCycler® 2.0 and a TaqMan™ assay on the ABI PRISM® 7900HT are shown in Table 7.3. Instruments capable of supporting rapid cycling protocols are increasingly available, although care should be taken to optimise the reaction and use appropriate reagents to maintain assay performance as rapid cycling may affect the sensitivity and precision of the assay.[43] The QuantiFast (Qiagen) system for rapid cycling utilises a buffer additive (Q-bond) to significantly reduce annealing, denaturation and extension times.

The choice of either two or three temperature cycles depends on the type of probe and instrument selected; TaqMan™ probe assays use a combined annealing and extension, molecular beacon assays use a three-stage reaction, while Scorpion™ and hybridisation probe assays may use either two or three temperature cycling. In real-time PCR most amplicons are typically short, so the requirement for the polymerase to extend at the optimum temperature is

Table 7.3 Typical reaction conditions for two common instrument and assay combinations.

	SYBR® Green in LightCycler® 2.0	*TaqMan™ probe assay on ABI PRISM® 7900HT*
Reaction	5–20 µl volume in glass capillaries	25–50 µl volume in 96 well plate
Master mix	1 × master mix, 1–5 mM $MgCl_2$, 1 mM dNTPs, 1 U DNA polymerase	1 × master mix, 3–10 mM $MgCl_2$, 1 mM dNTPs, 1–3 U DNA polymerase
Primers	0.5 µM each (0.1–0.8 typical range)	0.5 µM each (0.1–0.8 typical range)
Probe/dye	1:10 000 dilution SYBR® Green I	100–900 µM probe
Initial denaturation	95 °C 30 s	50 °C 2 min (UNG reaction) 95 °C 10 min (hot start)
Cycling	25–60 cycles	25–50 cycles
Denaturation	95 °C 0 s	95 °C 15 s
Primer annealing*	55 °C 5 s	60 °C 1 min
Extension	72 °C 10 s	
Data acquisition	End of extension step	During annealing/extension
Melt curve analysis	95 °C 0 s 35 °C 2 min 35 °C–95 °C at 0.1 °C/s	

* The primer annealing temperature used will be determined by the primer sequences used in the specific assay.

not absolute. Thus a two-step protocol is recommended in TaqMan™ probe assay, for example, as combining the annealing and extension stages into one step is quicker than the three steps (less time is taken during ramping between hold steps), and the relatively high annealing temperature ensures reaction specificity.

The melting temperature of oligonucleotide primers and probes determines the annealing temperature, or the combined annealing/extension temperature, at which fluorescence acquisition is performed. Assays that are designed to detect the presence of DNA sequences should possess annealing temperatures that are lower than the T_ms of the primers and probes. However, selection of annealing temperature is more complicated when target detection and discrimination is required, as ideally at the fluorescence acquisition temperature the oligonucleotide probe should be hybridised to perfectly matched sequences but should not be hybridised to mismatched targets, thus only generating signal from the fully complementary target. Optimisation of the annealing temperature is required to maximise the quantity of fluorescent signal emitted from matched probe whilst minimising the amount of mismatched probe that is hybridised. Achieving a reliable discriminatory assay using signal accumulation may not be possible if the matched and mismatched probe duplexes exhibit only small differences in T_m (less than 1–2 °C).

The hold times required in PCR cycles are determined by the type of polymerase, probe and instrument utilised. For example, if the *Z-Taq*™ enzyme and the rapid cycling conditions of the LightCycler® are used to amplify target sequences, denaturation, annealing and extension hold times may be reduced to 0 seconds, such that a 40 cycle amplification takes approximately 10 minutes to complete. The extension temperature of PCR may also affect the hold time required. At 72 °C, *Taq* polymerase adds significantly more nucleotides per second to extending products than at the 58–65 °C combined annealing/extension temperatures employed in two-stage PCR cycles. The hold time for PCR extension also depends on the size of the product being amplified, as larger targets require longer extension times.

One further consideration is to ensure that the excitation of the fluorophore is not too high, resulting in irreversible photobleaching and loss of fluorescent signal. Utilising less labile fluorophores, or performing the first ten cycles of amplification without fluorescence acquisition, can minimise loss of signal.

7.4.4 Primer and Probe Optimisation

A number of parameters will require optimisation in developing a robust assay, including the concentration of the primers, the amount of probe/intercalating dye reporter, the $MgCl_2$ concentration and the annealing temperature as already discussed. The performance of the assay is usually tested at a range of primer concentrations, from 50–900 nM using each primer at each concentration. The combination of concentrations yielding the lowest Ct is chosen (Figure 7.8).

When fluorescent oligonucleotide probes hybridise to target sequences during PCR, they must compete with the product' homologous strand. Unequal amounts of each primer may be used in an assay to generate effectively single-stranded targets, which can enable more efficient probe hybridisation since the concentration of the competing homologous PCR strand is significantly reduced. The primer that generates the homologous PCR strand is used at a significantly reduced concentration, where ratios between the two primers are typically between 10:1 and 100:1.[44] The quality of melt curve peaks may also be improved by the reduction in the amount of competing homologous product strands, although as a reduced amount of product is generated the efficiency of target detection may be reduced in some assays.

Using the optimal primer concentrations the probe/reporter is then optimised for each assay (Figure 7.9). The concentration of fluorescent probe affects the signal-to-noise ratio, so should be optimised such that the signal emitted from unhybridised probe is low or negligible, but the signal emitted from hybridised probe is significant. If too little probe is used in PCR assays, the amount of background signal is small but the amount of fluorescent signal produced upon hybridisation is also small. However, if too much probe is employed, the background fluorescence emitted from unhybridised probe will be large and may obscure the signal generated by hybridisation. The probe

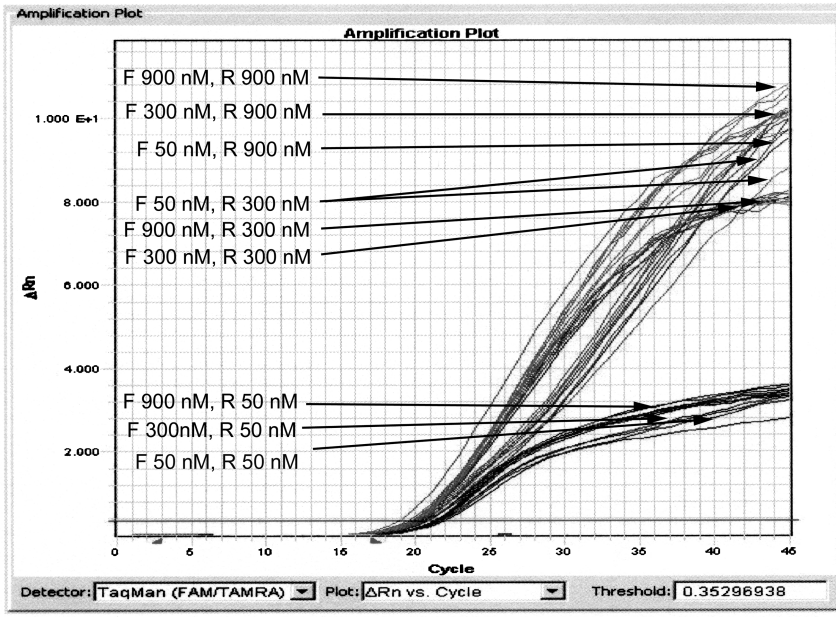

Figure 7.8 Results of a typical experiment optimising the concentration of PCR primers for a TaqMan™ assay. Manufacturer guidelines recommend that a variety of forward (F) and reverse (R) primer concentrations, usually from 50–900 nM as shown here, are tested in combination, to determine the optimal concentration for the assay. In this experiment, the 300 nM forward and 300 nM reverse combination was chosen as the optimal, being the lowest concentrations that reproducibly yielded the earliest Ct values whilst retaining a sigmoidal curve. It can also be seen that the change in reverse primer concentration has more effect on the kinetics than changes in the forward primer.

concentration also affects the hybridisation kinetics; to ensure that probe hybridisation occurs with a high efficiency and that there is sufficient probe to bind and detect the large amounts of target generated during amplification a molar excess of probe should be included in PCR reactions. However, the concentration of probe should not be sufficient to generate large background signals or to cause inhibition of the PCR.

For assays utilising fluorescent intercalating dyes as reporters, the dye concentration similarly requires optimisation for each set of PCR primers. Typically SYBR® Green I is used at a 1:10 000 dilution of the stock concentration, although higher concentrations (1:7000) may inhibit the enzyme.[45]

7.4.5 Target Level

In homogeneous assays, utilising fluorescence emission to monitor product accumulation, insufficient template may not generate increases in fluorescent

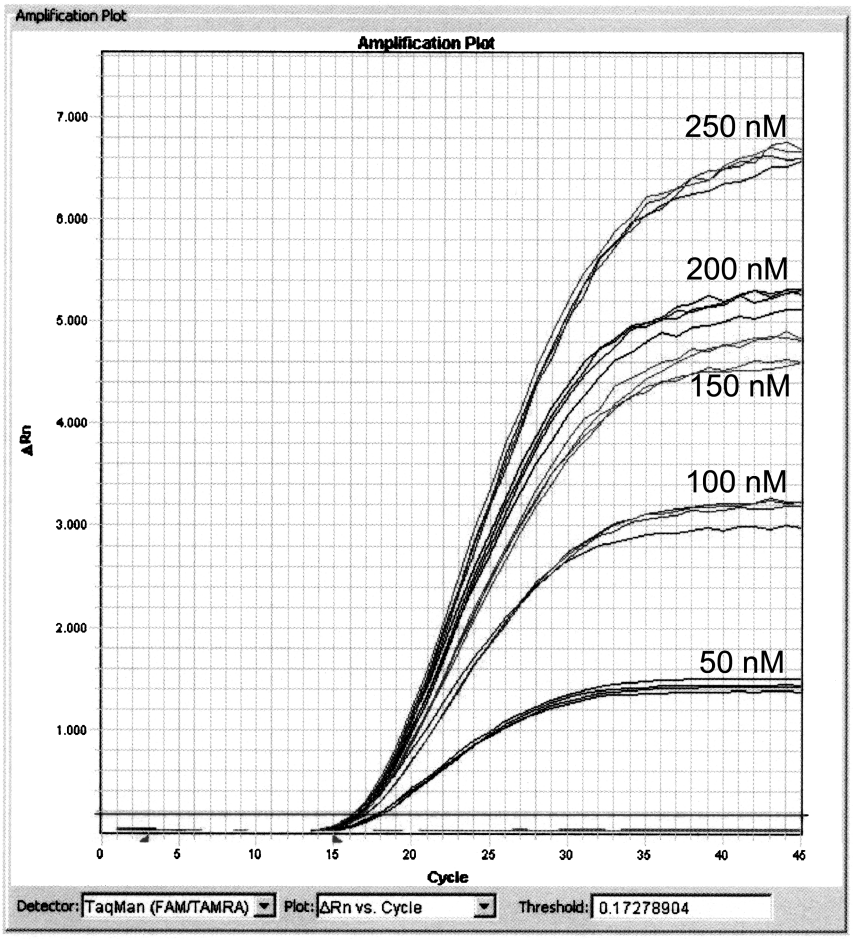

Figure 7.9 Experiment showing optimisation of a TaqMan™ probe for a typical assay. Manufacturer guidelines recommend testing a range of probe concentrations with the optimal primer levels, to determine the minimum effective amount of probe required in each assay. Here concentrations from 50 to 250 nM were tested. The 250 nM concentration was identified as yielding the highest relative fluorescence intensity, so was chosen for future use to ensure optimal assay performance.

signal during the course of amplification, whilst excess target may promote fluorescence increases prematurely in the reaction (Figure 7.10), and may also promote the generation of non-specific products. If fluorescence signal from reactions with very high template concentrations crosses the threshold value in the early cycles the baseline settings will be affected, although this can be rectified by manually setting the baseline to be calculated before any signal is detected. Typically, the quantity of genomic DNA included in homogeneous

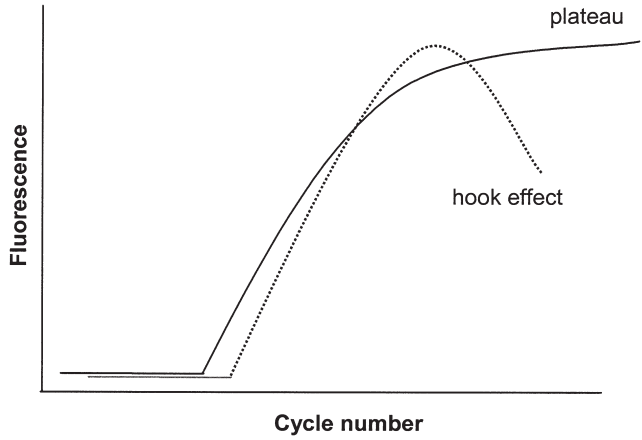

Figure 7.10 Schematic diagram illustrating the 'hook effect' often observed at high template concentrations.

assays is between 10 ng and 200 ng, although the concentration of unknown samples in quantification assays cannot always be controlled. Special considerations required when determining the amount of trace levels of target are discussed in more detail in Section 7.5.

In some experiments, a decrease in fluorescent signal is observed following the exponential phase of PCR. This 'hook effect'[46] is presumed to derive from competitive hybridisation between the single strands of the PCR product and the oligonucleotide probe. At low product concentrations, oligonucleotide probes compete efficiently for hybridisation target sites and, therefore, fluoresce efficiently. However, when the amount of PCR product is high, the two PCR strands re-anneal faster than the oligonucleotide probes can hybridise to their target sequence, such that the amount of fluorescence emission decreases. The observed decrease in fluorescence does not affect the efficiency or specificity of amplification or target detection, although optimising DNA template and $MgCl_2$ concentrations and reducing the number of PCR cycles performed, or utilising asymmetric target amplification,[47] may reduce the effect.

7.4.6 Contamination Control

As with standard PCR (Chapter 6), care is needed to ensure amplification reactions are not contaminated with exogenous targets such as amplification products from previous reactions. Homogeneous PCR assays generate significantly reduced amounts of post-amplification contaminant compared with conventional methods, since post-PCR product manipulation is not required. However, precautions such as the use of dedicated DNA-free PCR set-up areas and equipment, wearing appropriate protective clothing and using aerosol-resistant pipette tips are all still recommended. In addition, many commercial real-time master mixes include dUTP in the nucleotide mix and uracil

N-glycosylase (at approximately 0.01 U μl^{-1}) to clear any dUTP-containing PCR products carried over into the reaction.

7.4.7 Experimental Design

As described in Chapter 2, several factors should be considered in designing an experiment, including the appropriate level of replication, the controls to include and the need for randomisation of the samples, standards and controls. Depending on the instrument used the number of reactions that can be performed in one run can be limited (the LightCycler® 2.0, for example, has a 32 reaction capacity), and thus the appropriate choice of samples, controls and calibrants is vital to be able to interpret experimental results confidently.

7.4.7.1 Use of Controls

Negative controls should always be included, ideally both PCR reaction set-up controls and also negatives that have been subjected to the same extraction and preparation processes as the samples being analysed. It is advisable to run a number of negative controls and to intersperse their preparation with that of the unknown samples to obtain a representative estimation of the level of contamination in the analytical process. Without the inclusion of such controls it is impossible to determine if signals arise from the amplification of endogenous sample targets, or if cross-contamination between samples has occurred.

The use of positive controls is also desirable, to ensure the reaction components are functional and that the efficiency of the assay is acceptable. Internal positive control reactions can also demonstrate that no reaction inhibition has occurred,[48] which is especially important in the interpretation of apparently negative results from clinical or environmental samples. Inclusion of characterised positive samples can also be used to compare with unknown samples in post-amplification melt curve analysis, and are useful in assessing reaction specificity in genotyping assays.

7.4.7.2 Level of Replication

As discussed in Chapter 2, performing sufficient replicate measurements of an unknown sample can increase the confidence with which the quantitative data are interpreted, and is often crucial in providing sufficient analytical sensitivity for trace level analytes (Section 7.5). For quantitative determinations, six replicates are recommended to obtain a result with a low associated coefficient of variance. However, constraints of sample availability, time or cost may necessitate some reduction from this ideal. Studies of current practice in several sectors from 2004–2006 revealed that the laboratories questioned included between four and nine points in standard curves, and performed an average of three replicate reactions for both DNA standards (range 2–3) and unknown samples (range 2–8).

7.4.7.3 *Randomisation*

Again depending on the instrument used, it can be desirable to randomise the arrangement of samples to avoid any amplification bias resulting from temperature differences between the reaction positions. Instruments such as the Corbett Rotor-Gene™ rotate the samples during the reaction, so ensuring thermal homogeneity of the reactions. However, we have noted that the thermal cycling block on the ABI PRISM® 7700 SDS has a temperature differential, which can affect the efficiency of the reaction under certain circumstances (Figure 7.11).

The advent of higher-throughput robotic systems is facilitating the use of more complex experimental designs, such as the use of randomised plate layouts.

7.4.8 Data Analysis

Real-time PCR instruments are generally provided with manufacturer specific software, and thus the method of deriving the concentration or copy number of unknown samples from the fluorescent amplification signals may vary depending on the instrument and software version used. In addition, a number

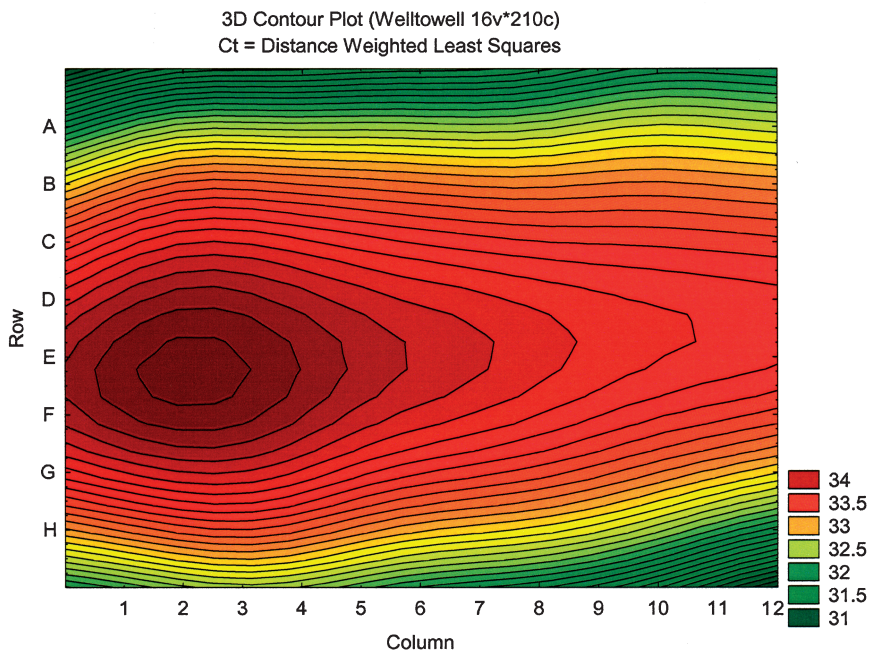

Figure 7.11 Contour plot representation of the results of 96 identical reactions set up on an 8×12 well microtitre plate, run on the ABI PRISM® 7700. The key shows the Ct values observed across the plate. Sub-optimal probe concentrations were used to enable differences in reaction efficiency due to temperature variations to be determined.

of quantification approaches have been detailed in the literature. It is not surprising that difficulties in data comparison may arise, despite having the same mathematical fundamentals.[49]

7.4.8.1 Basic Mathematics of PCR Amplification

The PCR process generates anywhere between an average of 0 to 1 copy of each target in each reaction cycle, so that for any cycle the number of molecules is:

$$N_C = N_{C0} \times (E + 1)^C \tag{7.1}$$

where N_C is the number of molecules at cycle N, N_{C0} is the number of molecules at cycle 0, E is the efficiency of the reaction and C is the cycle number. Making assumptions that the efficiency of the amplification is constant in the early exponential stages of the reaction, and that all standards possess the same number of target molecules at the point at which their signal crosses the determined threshold level, then the equation may be simplified to allow determination of the reaction efficiency, E_S (Equation (7.2)).

$$E_S = 10^{-\text{slope}} - 1 \tag{7.2}$$

The number of molecules at the threshold point, N_t, can also be determined from the standard curve (Equation (7.3)).

$$N_t = 10^{\text{Intercept}} \tag{7.3}$$

7.4.8.2 Data Normalisation

In many instruments each individual reaction position is excited and detected independently. In addition, in any instrument there is the potential for variability between reactions caused by small differences in pipetting. To overcome potential variation between reactions, it is possible to incorporate a passive reference dye, commonly ROX, in each reaction. The signal is monitored from the passive reference during the course of the amplification, and each well is then normalised using the unchanging reference signal. An advantage of the normalisation strategy is that the impact of any optical variability across the instrument will be minimised, but as an additional fluorescence channel is used to make the measurements the multiplexing capacity is reduced.

The raw data produced during amplification are also usually corrected to remove background noise from the measurements. It is usually possible to either set a manual baseline or to use settings calculated by the instrument. Ideally assays should be designed with a low initial signal and a significant increase resulting from amplification, to enable effective noise reduction.

A more complex normalisation approach, including curve smoothing and amplitude normalisation, has also been described to facilitate gene expression determination by a standard curve approach.[50]

7.4.8.3 Routes to Determining Amplification Efficiency

The threshold method is most commonly used for the quantification of unknowns, and as illustrated in Figure 7.1 utilises information from the points at which known DNA standards reach a specified fluorescence threshold to construct a standard curve of crossing threshold against target level. As described, the reaction efficiency is determined from the slope of the standard curve. The threshold level should be set in the exponential phase of the amplification, and most instruments calculate an optimal level setting. Manually setting the level is possible, but is subjective and may also introduce variability between runs of the assay. Advantages are that the method is simple, and the quality of the assay may be monitored using the parameters of the standard curve. Disadvantages are that the dilution series used to construct the standard curve is prone to errors, and the assumption that the reaction efficiency is a constant in the exponential phase of the reaction is not always valid.[51]

To overcome the problems in using a dilution series, alternative methods based on estimating the amplification efficiency from single reactions have been developed.[52–54] The rate of change of fluorescent signal within a single reaction may be monitored, ideally within the linear phase of signal increase, to determine the efficiency of each reaction. The second derivative maximum option in the LightCycler® software similarly calculates the maximum rate of change of the signal in the reaction, and utilises the peak to determine the fluorescence at the maxima, and hence the initial number of copies in the reaction.

A third mathematical approach utilising branching process theory to model amplification and determine reaction efficiencies has also been developed and validated using qPCR data,[55–56] and reflects the stochastic nature of the process.

7.4.8.4 Outlier Identification

Other mathematical treatment of data that can benefit the accuracy of the results obtained is identification of outliers. In ISO 5725 guidelines, outliers are classed as results which lie beyond 99% of the range of the characterised distribution (those which have a probability value less than 1%). Inclusion of such inconsistent data points can affect quantitative results, and ideally should be identified and omitted from the analysis. A number of routes to outlier identification have been developed, including detecting dissimilarities of amplification efficiencies of replicate reactions[53,57] and the use of the Grubbs test to assess Ct values.[58]

7.4.9 Validation

It may be necessary to validate a newly developed or introduced qPCR assay, to determine the scope and performance of the method in-house. The approach to method validation is described in detail in Chapter 3 and so will not be

Table 7.4 Parameters and approaches to consider in assessing qPCR performance.

Performance Characteristic	*qPCR performance*	*Experimental procedure*
Dynamic range	Range of sample concentrations over which the assay remains linear	Dilution series from a known concentration DNA analysed
Repeatability	Variability of result under closely controlled conditions	Same measurements on same sample repeated by same analyst
Reproducibility	Variability of result under differing conditions	Same sample measurement repeated by different analysts using different instruments, different laboratories or over time
Bias	Consistent over- or underestimation of the true result	Average measured value of a reference material compared to the assigned value
Specificity	Ability of the assay to detect the target but not other potential analytes present in the sample	Assay performed with a variety of related targets to check for false positive signals
Sensitivity (LOD/ LOQ)	The lowest amount of the target that is detectable/ reliably quantifiable	Assay performed with increasing dilutions of analyte to determine the limit of detection/linearity

repeated here, other than to highlight the performance characteristics that might be usefully assessed (Table 7.4).

The expected range for both correlation coefficient and slope of the standard curve for quantitative assays can also be determined. For TaqMan™ probe assays, with typical reaction efficiency values between 92 and 110%, the slope range would be from approximately –3.52 to –3.1. However, acceptable correlation coefficient and slope ranges may be set by the laboratory according to the needs of the application. These values can then be compared to the actual assay performance over time as measures of acceptable quality, enabling problems or errors to be identified.

7.5 Quantification of Low Levels of Target Analyte

Accurate quantification of trace amounts of DNA targets using qPCR is increasingly important for clinical, environmental and forensic applications,

although instrument manufacturers generally do not recommend quantification of less than 5000 target copies.

7.5.1 Level of Variability

At high target concentrations the CV is usually less than 1%, however at low target levels the accuracy of measurement is sensitive both to target losses during sample preparation and to high levels of sampling variability. A number of studies[41,59] have shown that analytical variability increases with decreasing copy number (Figure 7.12).

7.5.1.1 Sample Handling

Solutions containing very low concentrations of target can be significantly affected by DNA sticking to tube walls during preparation stages, probably as a larger proportion of the total is lost. Sample loss may be minimised by using low retention or siliconised plastic ware, and preparing low concentration standards just prior to reaction set-up rather than storing dilute solutions.[41,61]

It is also recommended to use volumes of $\geq 250\,\mu l$ when preparing standard curve DNA dilutions for analysis, as using smaller volumes can decrease both sensitivity and precision.[62] The difference in sensitivity may be attributable either to sampling variation or to the greater surface area of the dilution solution that is in contact with the tube in the low volume dilutions. Despite the use of siliconised plasticware some such losses may still be expected, and the

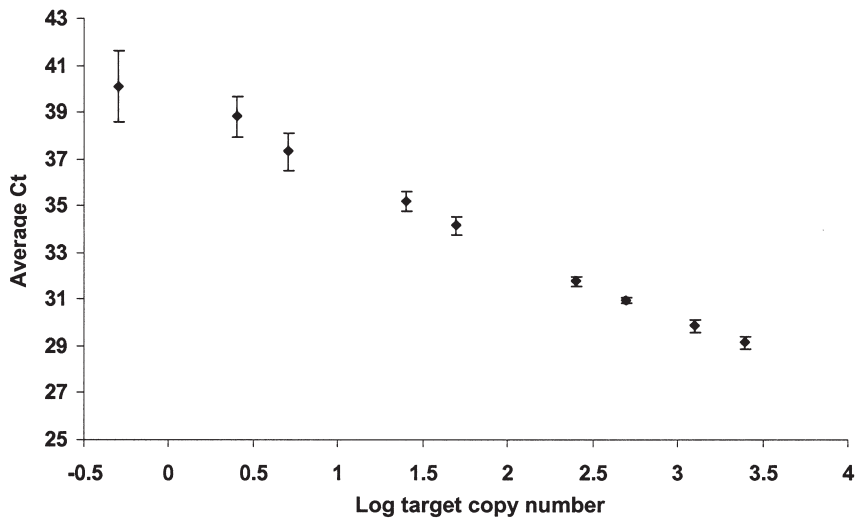

Figure 7.12 Increased variability at low target levels. The average Ct (±1 SD) values are plotted against log target copy number. The results were obtained from 12 repeat standard curves from an SRY assay[60] with all points measured in triplicate, and reactions yielding a Ct ≥ 55 excluded.

larger contact area may lead to a greater proportion of the target copies being lost through adsorption.

7.5.1.2 Amplification Cycles

Typically in the region of 30 cycles are performed in a qPCR analysis, yielding a clear measurable signal. However, assays designed to detect and quantify even very small numbers of targets can benefit from extending the number of cycles performed. Utilising a longer reaction with 55–60 cycles is of benefit both by permitting detection of samples that only reach detectable fluorescence levels late in the reaction and by maximising the difference between true negative and late-appearing positive signals (Figure 7.13). Clear distinction between reaction negative controls and positive signals can significantly increase confidence in late-appearing signals, thus increasing the effective sensitivity of the analysis.

7.5.1.3 Replication Level

Increasing the number of replicates performed can improve the effective sensitivity of an assay detecting very low concentration analyte, by raising the probability that the target will be sampled from the bulk solution. This increased sensitivity has been utilised in the clinical setting,[63] and can also be modelled using logistic regression (Figure 7.14) to determine the expected probability of target detection of an assay for a given analyte concentration.[61]

7.5.1.4 Data Handling

At very low target levels the significant proportion of replicate reactions that fail to yield a measurable Ct present an additional challenge to achieving reliable analysis. Sampling variability results in a number of low copy number reactions without any target, which consequently do not yield a signal. Such reactions preclude the use of normal statistical analysis as there is no meaningful Ct value that can be assigned. In addition, simply ignoring the

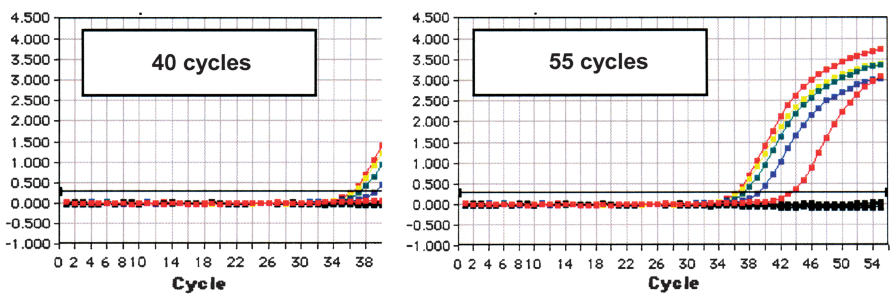

Figure 7.13 Amplification plots of SRY targets from human male genomic DNA on the ABI PRISM® 7700. Data from 40 and 55 cycle qPCR reactions, demonstrating the increased level of information obtained from very low target concentration samples (0–15 genome equivalents per reaction).

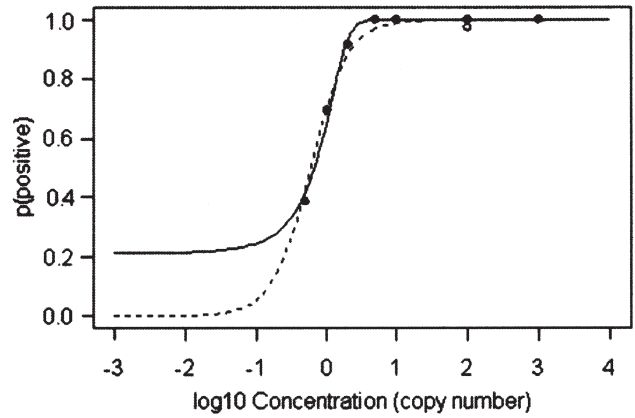

Figure 7.14 Logistic regression of detection probability.[61] Logistic regression of p(positive) *vs.* concentration C with false negative at $\log_{10}(C)$ omitted (solid line) and p(positive) *vs.* $\log_{10}(C)$ with false negative included (dashed line). Solid points show fraction of positives at each concentration with the false negative omitted; the open circle shows the calculated fraction at $\log_{10}(C)$ with the false negative included. The apparent lower limit to the solid curve is an artefact of plotting on the $\log_{10}$ axis.

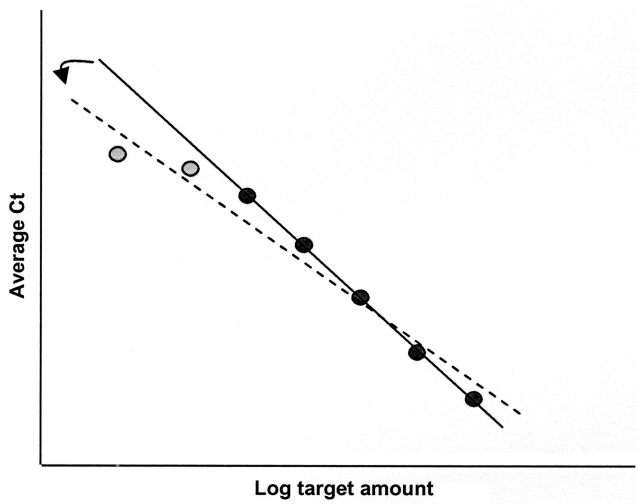

Figure 7.15 Schematic demonstrating the potential bias from omitting negative replicates and underestimating low standard concentration Ct values.[61]

replicate amplification failures is effectively omitting information from the assay, which can introduce bias into the results by assigning a lower average Ct to standards or samples than is warranted by the full data.[61] The lower Ct values can skew the regression line of the standard curve, and affect the quantification results at all target concentrations (Figure 7.15). To avoid bias

in the analysis the standard curve should be constructed only using standard dilution points where all replicate reactions yield a meaningful measurable Ct value. In addition, to determine the concentration value of unknown samples average Ct values should not be used. Each replicate Ct should be separately interpolated to yield a concentration or copy number value, and any replicate reactions with no detectable signal due to sampling variability at very low concentration can be assigned a value of zero. Then the concentration of target in the unknown sample may be determined by averaging the replicate reaction values.

7.6 Standards and Comparability

The development of qPCR has enabled performance of quantitative measurements with very low associated variability, with CVs of between 2 and 5% commonly reported in the literature. However, there are many potential sources of analytical error in the process, ranging from operator-introduced variability,[64] temperature or detector heterogeneity in the instrument, variations in reagent integrity and activity, effects of target melting behaviour[65] and inherent variations in the PCR amplification itself. The combination of these factors can have a significant cumulative effect on quantitative results obtained, which may be further exacerbated by the exponential nature of the reaction. Consequently it is desirable for users of the technique to understand the sources of experimental variability, in order to control and/or monitor for potential problems.

7.6.1 Quantitative Standards

The accuracy of any qPCR analyses that utilise DNA standards or calibrators to anchor the quantitative measurements is largely dependent on the quality of the standard used. Commonly DNA standards are prepared and quantified in-house, largely because suitable reference materials are not yet commercially available. The quantification of DNA standards may vary significantly between laboratories and methods, as discussed in more detail in Chapter 5. Thus the ability to perform 'absolute quantification' using real-time PCR analysis is practically limited by the certainty with which the concentration of the DNA standard is known. However, the software provided with many real-time instruments generates concentration or copy number results to several decimal places, implying a degree of analytical accuracy that is unlikely to be achieved in practice.

Of perhaps more concern is that the approximate DNA concentrations assigned to many commercial DNA preparations are sometimes used as accurate concentration values by researchers. The downstream analyses are thus often reliant on concentration values which are neither intended nor suitable for the purpose. In the absence of certified quantitative reference materials and standards, an awareness of the limitations of quantitative accuracy is important to ensure results are understood and interpreted correctly.

Assays that depend on relative quantification are unaffected by the lack of quantitative DNA standards, as the ratio of two targets within the assay is assessed. If both targets are detected within the same assay, then many of the sources of measurement uncertainty similarly affect both determinations, and thus do not influence the final result. As an example, assays designed for the relative quantification of genetically modified organism (GMO) utilise the ratio of Ct values of endogenous and GM sequences. For this application reference materials are available containing a certified percentage GM, which are typically used as comparative standards underpinning confidence in the assay.

7.6.1.1 Instrument Calibration

Variability may be introduced by non-uniformity within the thermal cycler itself, depending on the instrument design. Consequently it is recommended to check the instrument performance regularly, to ensure the machine has an established calibration schedule and that any maintenance recommended by the manufacturer is carried out. Several commercial dye calibration kits and systems are currently available, which are useful to ensure that the selectivity of signal detection is set correctly. In addition, checks to ensure that the well or rotor positions are not contaminated with extraneous fluorescent dye and that the instrument is detecting fluorescence data at the correct positions are possible on some machines.

In addition it is possible for the thermal uniformity of heating blocks to be assessed by accredited testing laboratories, and a system for interim checks is also now available (the DRIFTCON® system from Anachem).

7.6.1.2 Comparability

Although qPCR exhibits low levels of variability when performed within a laboratory, comparative performance between laboratories is also required for many applications. Comparable performance may be achieved by using standardised methods and data handling approaches, calibrated equipment and reference materials where available. Several inter-laboratory performance studies have been performed utilising real-time PCR, and the results have demonstrated that there is appreciable variability between results obtained in different laboratories.[66,67] An early study of 42 clinical diagnostic laboratories found variation in quantitative accuracy, assay precision and sensitivity. In a later study of over 130 laboratories using a TaqMan™ based assay, requiring RNA extraction, reverse-transcription and qPCR, about one in five laboratories submitted one or more unsatisfactory results.[66]

Figure 7.16 shows the results of a quantitative TaqMan™-based assay performed by independent analysts as part of a proficiency testing scheme. The reported results varied by over an order of magnitude, highlighting the potential variability in quantitative results generated by different laboratories and analysts.

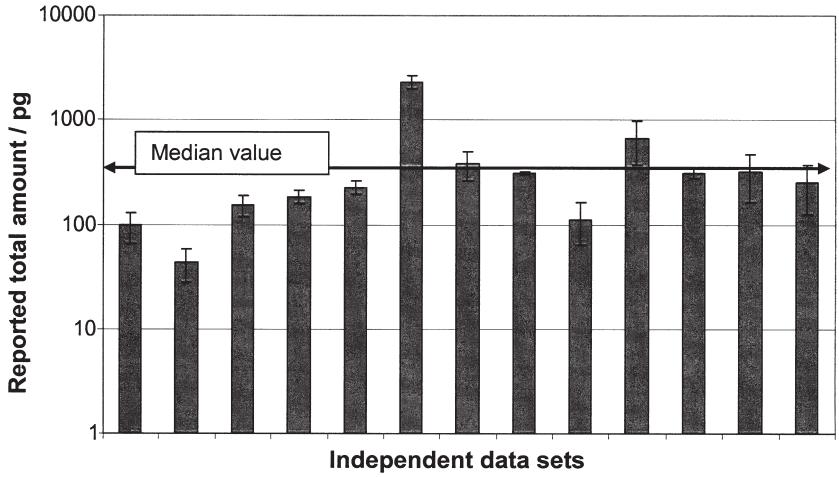

Figure 7.16 Results from one round of a PT scheme using a TaqMan™-based qPCR assay. The data have been normalised to exclude variability arising from DNA standard measurements, and reflect the average values of three identical samples provided to participants. The error bars represent ±1 SD of the three results for each participant, and the median value is shown as a solid line.

7.6.1.3 Measurement Uncertainty

Increasingly there is a requirement for analytical data to be reported with an associated measurement uncertainty value, as introduced in ISO 17025:2005. Detailed information on the calculation of uncertainty for a method can be found in the ISO Guide to the Expression of Uncertainty in Measurement (GUM). The need for determination of the uncertainty associated with a quantitative measurement arises because all measurements are estimations in reality, because it is not possible to completely control all possible sources of variability in an analysis to obtain a 'true value'. In summary the variability for each individual stage of the qPCR process should be determined, documented and combined to produce an overall uncertainty budget. In practice this is difficult to achieve, and estimation may be made from validation data and inter-laboratory comparisons.

7.7 Summary

As PCR has developed from a research to an analytical tool there has been an increasing demand for the provision of accurate quantitative data. Despite variation in the details of the approaches and assays that have been developed, certain principles underpin all such analyses in striving to achieve analytical accuracy. The use of appropriate controls and standards is essential in the quantitative process and should be selected to control for sample type and history, and be equivalent in terms of amplification potential. Careful assay

design in order to ensure equivalence of analytes and standards, and care in performing each assay to minimise potential variability resulting from pipetting, data analysis and documentation, will additionally serve to improve precision. Whilst technological improvements have been made allowing quantitative PCR measurements to be approached by the analytical community, the persisting challenge to the analyst is to demonstrate the accuracy of such measurements. Provision of suitable standards, certified reference materials and other QA tools such as appropriate accessible proficiency trials may help overcome current problems, and allow qPCR to fulfil its full analytical potential.

References

1. G. Gilliland, S. Perrin, K. Blanchard and H. F. Bunn, *Proc. Natl. Acad. Sci. USA*, 1990, **87**, 2725.
2. D. A. Levinson, J. Campos-Torres and P. Leder, *J. Exp. Med.*, 1993, **178**, 317.
3. M. J. Alvarez, A. M. Depino, O. L. Podhajcer and F. J. Pitossi, *Anal. Biochem.*, 2000, **287**, 87.
4. R. Higuchi, G. Dollinger, P. S. Walsh and R. Griffith, *Biotechnology (NY)*, 1992, **10**, 413.
5. http://www.stratagene.com/lit_items/TN5_Standard_Curves_technotefinal.pdf (accessed May 2007).
6. L. G. Lee, C. R. Connell and W. Bloch, *Nucl. Acids Res.*, 1993, **21**, 3761.
7. P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 7276.
8. K. J. Livak, S. J. Flood, J. Marmaro, W. Giusti and K. Deetz, *PCR Meth. Appl.*, 1995, **4**, 357.
9. K. J. Livak, *Genet. Anal.*, 1999, **14**, 143.
10. http://www.appliedbiosystems.com/ (accessed May 2007).
11. I. V. Kutyavin, I. A. Afonina, A. Mills, V. V. Gorn, E. A. Lukhtanov, E. S. Belousov, M. J. Singer, D. K. Walburger, S. G. Lokhov, A. A. Gall, R. Dempcy, M. W. Reed, R. B. Meyer and J. Hedgpeth, *Nucl. Acids Res.*, 2000, **28**, 655.
12. C. Letertre, S. Perelle, F. Dilasser, K. Arar and P. Fach, *Mol. Cell. Probes.*, 2003, **17**, 307.
13. S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303.
14. http://www.molecular-beacons.com/default.htm (accessed May 2007).
15. S. Tyagi, D. P. Bratu and F. R. Kramer, *Nat. Biotechnol.*, 1998, **16**, 49.
16. D. J. French, C. L. Archard, T. Brown and D. G. McDowell, *Mol. Cell. Probes.*, 2001, **15**, 363.
17. M. Lee, S. Siddle and R. Hunter, *Anal. Chim. Acta*, 2002, **457**, 61.
18. C. T. Wittwer, M. G. Herrmann, A. A. Moss and R. P. Rasmussen, *Biotechniques*, 1997, **22**, 130.
19. P. S. Bernard, R. S. Ajioka, J. P. Kushner and C. T. Wittwer, *Am. J. Pathol.*, 1998, **153**, 1055.

20. D. Whitcombe, J. Theaker, S. P. Guy, T. Brown and S. Little, *Nat. Biotechnol.*, 1999, **17**, 804.
21. S. C. Johnson, C. B. Sherrill, D. J. Marshall, M. J. Moser and J. R. Prudent, *Nucl. Acids Res.*, 2004, **32**, 1937.
22. H. A. Cubie, A. L. Seagar, E. McGoogan, J. Whitehead, A. Brass, M. J. Arends and M. W. Whitley, *Mol. Pathol.*, 2001, **54**, 24.
23. S. D. Dufresne, D. R. Belloni, W. A. Wells and G. J. Tsongalis, *Arch. Pathol. Lab. Med.*, 2006, **130**, 185.
24. http://bio-rad.com (accessed May 2007).
25. http://www.stratagene.com (accessed May 2007).
26. http://www.techne.com (accessed May 2007).
27. http://www.biogene.com (accessed May 2007).
28. http://biochem.roche.com (accessed May 2007).
29. http://www.cepheid.com/Sites/cepheid/section.cfm?id=1 (accessed May 2007).
30. http://www.corbettlifescience.com (accessed May 2007).
31. L. J. Kricka and P. Wilding, *Anal. Bioanal. Chem.*, 2003, **377**, 820.
32. http://web.ncifcrf.gov/rtp/gel/primerdb/ (accessed May 2007).
33. M. Zuker, *Nucl. Acids Res.*, 2003, **31**, 3406.
34. http://www.bioinfo.rpi.edu/applications/mfold/ (accessed May 2007).
35. http://www.ncbi.nlm.nih.gov/BLAST/ (accessed May 2007).
36. http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm (accessed May 2007).
37. http://www.autoprime.de/AutoPrimeWeb (accessed May 2007).
38. R. B. Wallace, M. J. Johnson, T. Hirose, T. Miyake, E. H. Kawashima and K. Itakura, *Nucl. Acids Res.*, 1981, **9**, 879.
39. Z. Guo, Q. Liu and L. M. Smith, *Nat. Biotechnol.*, 1997, **15**, 331.
40. J. G. Wetmur, *Annu. Rev. Biophys. Bioeng.*, 1976, **5**, 337.
41. I. A. Teo, J. W. Choi, J. Morlese, G. Taylor and S. Shaunak, *J. Immunol. Meth.*, 2002, **270**, 119.
42. K. A. Kreuzer, A. Bohn, U. Lass, U. R. Peters and C. A. Schmidt, *Mol. Cell. Probes*, 2000, **14**, 57.
43. C. Hilscher, W. Vahrson and D. P. Dittmer, *Nucl. Acids Res.*, 2005, **33**, e182.
44. S. K. Poddar, *Mol. Cell. Probes*, 2000, **14**, 25.
45. K. Nath, J. W. Sarosy, J. Hahn and C. J. Di Como, *J. Biochem. Biophys. Meth.*, 2000, **42**, 15.
46. Roche Molecular Biochemicals Technical note, No. LC 8/99, 1999.
47. K. Barratt and J. F. Mackay, *J. Clin. Microbiol.*, 2002, **40**, 1571.
48. M. Stöcher, V. Leb, G. Holzl and J. Berg, *J. Clin. Virol.*, 2002, **25**, Suppl 3, S47.
49. R. G. Rutledge and C. Cote, *Nucl. Acids Res.*, 2003, **31**, e93.
50. A. Larionov, A. Krause and W. Miller, *BMC Bioinformatics*, 2005, **6**, 62.
51. W. Liu and D. A. Saint, *Anal. Biochem.*, 2002, **302**, 52.
52. W. Liu and D. A. Saint, *Biochem. Biophys. Res. Commun.*, 2002, **294**, 347.

53. S. N. Peirson, J. N. Butler and R. G. Foster, *Nucl. Acids Res.*, 2003, **31**, e73.
54. A. Tichopad, M. Dilger, G. Schwarz and M. W. Pfaffl, *Nucl. Acids Res.*, 2003, **31**, e122.
55. J. Peccoud and C. Jacob, *Biophys. J.*, 1996, **71**, 101.
56. N. Lalam, C. Jacob and P. Jagers, *Adv. Appl. Probab.*, 2004, **36**, 602.
57. T. Bar, A. Stahlberg, A. Muszta and M. Kubista, *Nucl. Acids Res.*, 2003, **31**, e105.
58. M. J. Burns, G. J. Nixon, C. A. Foy and N. Harris, *BMC Biotechnol.*, 2005, **5**, 31.
59. J. Stenman and A. Orpana, *Nat. Biotechnol.*, 2001, **19**, 1011.
60. L. Birch, C. A. English, K. O'Donoghue, O. Barigye, N. M. Fisk and J. T. Keer, *Clin. Chem. (Washington, DC)*, 2005, **51**, 312.
61. S. L. Ellison, C. A. English, M. J. Burns and J. T. Keer, *BMC Biotechnol.*, 2006, **6**, 33.
62. C. English, L. Birch and J. Keer, LGC Report, 2003,**LGC/VAM/2003/057**, available at www.nmschembio.org.uk through free registration.
63. I. Hromadnikova, B. Houbova, D. Hridelova, S. Voslarova, J. Kofer, V. Komrska and D. Habart, *Prenat. Diagn.*, 2003, **23**, 235.
64. S. A. Bustin, *J. Mol. Endocrinol.*, 2002, **29**, 23.
65. J. Wilhelm, M. Hahn and A. Pingoud, *Clin. Chem. (Washington, DC)*, 2000, **46**, 1738.
66. S. C. Ramsden, S. Daly, W. J. Geilenkeuser, G. Duncan, F. Hermitte, E. Marubini, M. Neumaier, C. Orlando, V. Palicka, A. Paradiso, M. Pazzagli, S. Pizzamiglio and P. Verderio, *Clin. Chem. (Washington, DC)*, 2006, **52**, 1584.
67. C. C. Raggi, P. Verderio, M. Pazzagli, E. Marubini, L. Simi, P. Pinzani, A. Paradiso and C. Orlando, *Clin. Chem. Lab. Med.*, 2005, **43**, 542.