Chapter 8

Quantitative Reverse Transcriptase Polymerase Chain Reaction

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INTRODUCTION

Since the first documentation of real-time polymerase chain reaction (PCR),[1] it has been used for an increasing and diverse number of applications, including mRNA expression studies, DNA copy number measurements in genomic or viral DNAs,[2–7] allelic discrimination assays,[8,9] expression analysis of specific splice variants of genes[10–13] and gene expression in paraffin-embedded tissues,[14,15] and laser captured microdissected cells.[13,16–19] Therefore, quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) is now essential in molecular diagnostics to quantitatively assess the level of RNA or DNA in a given specimen. Q-RT-PCR enables the detection and quantification of very small amounts of DNA, cDNA, or RNA, even down to a single copy. It is based on the detection of fluorescence produced by reporter probes, which varies with reaction cycle number. Only during the exponential phase of the conventional PCR reaction is it possible to extrapolate back in order to determine the quantity of initial template sequence. The "real-time" nature of this technology pertains to the constant monitoring of fluorescence from specially designed reporter probes during each cycle. Due to inhibitors of the polymerase reaction found with the template, reagent limitation or accumulation of pyrophosphate molecules, the PCR reaction eventually ceases to generate template at an exponential rate (i.e., the plateau phase), making the end point quantitation of PCR products unreliable in all but the exponential phase. Examples of fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e., SYBR® Green) or sequence-specific probes (i.e., TaqMan® products or Molecular Beacons Probes). The automation of the reaction as a whole has enabled Q-RT-PCR assays to be easy to perform with higher sensitivity and more specificity.

This chapter introduces important aspects of Q-RT-PCR, including comparison between conventional PCR and Q-RT-PCR methodology, fluorogenic and sequence-specific probes, methods for quantitation of Q-RT-PCR products, common terminology, and a review of instrumentation.

UNDERSTANDING THE FUNDAMENTALS

Quantitative reverse transcriptase polymerase chain reaction is the reliable detection and measurement of products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR process. Holland and co-workers[20] demonstrated that the thermostable enzyme Thermus aquaticus (i.e., Taq) DNA polymerase had 5′ to 3′ exonuclease activity. This group also showed that cleavage of a specifically designed target probe during PCR by the 5′ nuclease activity of Taq polymerase can be used to detect amplification of the amplified product.[20] An oligonucleotide probe, which was designed to hybridize within the target sequence, was introduced into the PCR assay. This probe was labeled with ^{32}P at its 5′ end and was nonextendable at its 3′ end to ensure it could not act as a primer. Annealing of probe to one of the PCR product strands during the course of amplification generated a substrate suitable for exonuclease activity. Also, during amplification, the 5′ to 3′ exonuclease activity of Taq DNA polymerase (when the enzyme extended from an upstream primer into the region of the probe) degraded the probe into smaller fragments that could be differentiated from undegraded probe. This dependence on polymerization ensured that cleavage of the probe occurred only if the target sequence was being amplified. After PCR, cleavage of the probe was measured by using thin-layer chromatography to separate cleavage fragments from intact probe. The introduction of dual-labeled oligonucleotide fluorogenic probes allowed the elimination of post-PCR processing for the analysis of probe degradation.[21] The probe has a reporter fluorescent dye at the 5′ end and a quencher dye attached to the 3′ end. While the probe is intact, the close proximity of the quencher significantly decreases the fluorescence emitted by the reporter dye. A fluorescence signal is only emitted on cleavage of the probe, based on the fluorescence resonance energy transfer (FRET) principle.[22]

In the real-time quantitative TaqMan® assay, a fluorogenic nonextendable probe, termed the "TaqMan" probe, is used (Figure 8.1).[23] The probe has a fluorescent reporter dye attached to its 5′ end and a quencher dye at its 3′ terminus. If the target

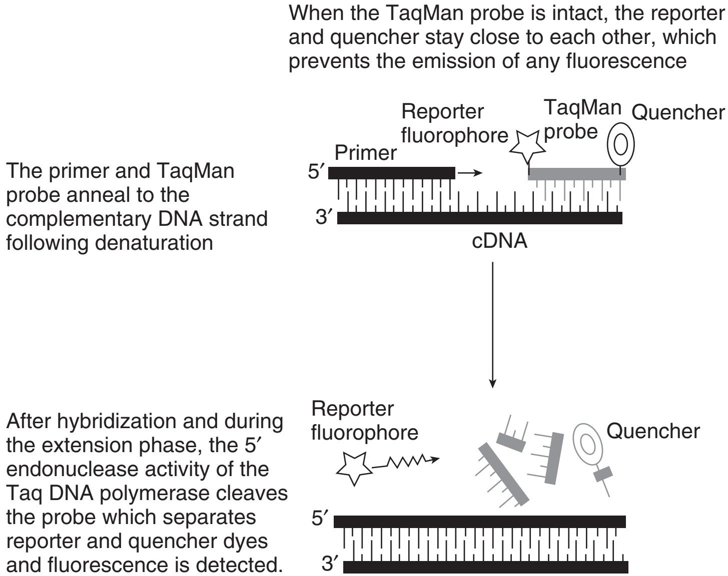

FIGURE 8.1. Hydrolysis probes (e.g., TaqMan assay). (From Ref. 73)

sequence is present, the fluorogenic probe anneals downstream from one of the primer sites and is cleaved by the 5′ nuclease activity of the Taq polymerase enzyme during the extension phase of the PCR. While the probe is intact, FRET occurs, and the fluorescence emission of the reporter dye is absorbed by the quenching dye. Cleavage of the probe by Taq polymerase during PCR separates the reporter and quencher dyes, thereby increasing the fluorescence from the former. Additionally, cleavage removes the probe from the target strand, allowing primer extension to continue to the end of template strand, thereby not interfering with the exponential accumulation of PCR product. Additional reporter dye molecules are cleaved from their respective probes with each cycle, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced. The various available chemistries for real-time PCR are described later in this review.

Using any of the developed chemistries, the increase in fluorescence emission during the PCR reaction can be detected in real time by a modified conventional PCR thermocycler. The computer software constructs amplification plots using the fluo-

rescence emission data that are collected during the PCR amplification (Figure 8.2). Figure 8.2 demonstrates a representative amplification plot and defines the important terms associated with it.

- Baseline: The baseline is defined as the PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument. By default, the computer software sets the baseline from cycles three to 15; however, this often needs to be changed manually utilizing software supplied with each particular thermocycler.
- ΔRn: A computer software program calculates a ΔRn using the equation Rn = Rnf – Rnb, where Rnf is the fluorescence emission of the product at each time point and Rnb is the fluorescence emission of the baseline.[23,24] The ΔRn values are plotted

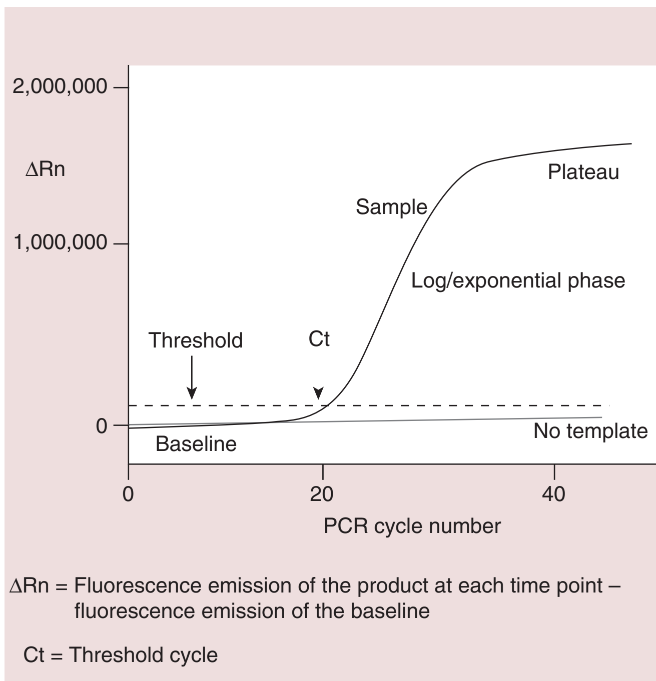

FIGURE 8.2. Model of a single amplification plot illustrating the nomenclature commonly used in real-time quantitative PCR. (From Ref. 73)

versus the cycle number. During the early cycles of PCR amplification, ΔRn values do not exceed the baseline.

- Threshold: An arbitrary threshold is chosen by the computers, based on the variability of the baseline. It is calculated as ten times the standard deviation of the average signal of the baseline fluorescent signal between cycles three to 15. A fluorescent signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. If required, the threshold can be manually changed for each experiment so that it is in the region of exponential amplification across all amplification plots.
- Ct: This is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the minimal detection level (i.e., the threshold). The Ct is a basic principle of real-time PCR and is an essential component in producing accurate and reproducible data.[1]

The presence of more template at the start of the reaction leads to a fewer number of cycles passing before reaching the point at which the fluorescent signal is recorded as statistically significant above background.[24] This Ct value will always occur during the exponential phase of target amplification, which occurs during the early cycles of PCR. As reaction components become limiting, the rate of target amplification decreases until the PCR reaction is no longer generating template at an exponential rate (plateau phase), and there is little or no increase in PCR product. This is the main reason why Ct is a more reliable measure of starting copy number than an endpoint measurement of the amount of accumulated PCR product. During the exponential phase, none of the reaction components is limiting, and therefore, Ct values are very reproducible for replicate reactions with the same starting copy number.

DISCUSSION OF METHODS TO QUANTIFY REAL-TIME POLYMERASE CHAIN REACTION RESULTS

Standard-curve or absolute quantitation: As shown by Higuchi and co-workers, the plot of the log of initial target copy number for a set of known standards (five- or tenfold serial dilution) versus Ct is a straight line (the standard curve).[1] Quantitation of the amount of target in the “unknown” samples of interest is accomplished by measuring Ct and using the standard curve to determine starting copy number. The most common source of a known sample is a plasmid for the gene of interest, and the standard curve is generated based on a serial dilution of a starting

amount. Another option, and easier to generate if a plasmid is unavailable, is the use of a synthetic single-stranded sense oligonucleotide for the entire amplicon. The advantage of this approach is that it significantly simplifies the process of obtaining a standard curve for amplicons up to 100 bp, which encompasses most real-time PCR amplicons. Furthermore, it is also less susceptible to bias when quantified by a spectrophotometer due to the relative purity of the oligonucleotide. Together with the greater precision of measurement of the standard and the possibility of calculating the moles of oligonucleotide (hence, number of copies); it is possible to approximate the number of copies of a template in an unknown sample, although not in terms of absolute copy number. One final option for a standard curve is to use a cell line with a known copy number or expression level of the gene of interest. The standard curve method is used in circumstances when absolute quantitation is critical for the investigator (e.g., when measuring a small number of genes in either a few or many samples)[25,26] or in quantitation of viral load.[27–29]

Relative quantitation: Relative quantitation is also known as the comparative threshold method (2-Ct method). This method eliminates the need for standard curves, and mathematical equations are used to calculate the relative expression levels of a target relative to a reference control or calibrator, such as a nontreated sample or RNA from normal tissue. The amount of target, normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by 2-Ct, where Ct = Ct (sample) − Ct (calibrator), and Ct is the Ct of the target gene subtracted from the Ct of the housekeeping gene. The equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator sample. For this calculation to be valid and in order to obtain reliable results, it is imperative that the amplification efficiencies of the housekeeping and target gene are approximately equal and at or above 90%. This can be established by looking at how Ct (of both sample and calibrator) varies with template dilution. If the plot of complementary DNA (cDNA) dilution versus Ct is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, the standard curve method is then preferable. Alternatively, new primers can be designed and/or optimized to achieve a similar efficiency for the target and housekeeping gene amplicons.

CONTROLS IN QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION: USE OF THE "HOUSEKEEPING" GENE

In real-time quantitative PCR experiments specific errors will be introduced due to minor differences in the starting amount of RNA, quality of RNA, or differences in efficiency of cDNA synthesis and PCR amplification. In order to minimize these errors and correct for sample-to-sample variation, a cellular RNA is simultaneously amplified with the target, which serves as an internal reference against which other RNA values can be normalized. The most common genes used for normalization, termed "housekeeping" genes, are β-actin, a cytoskeletal protein, and glceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme,[30] and ribosomal RNA (rRNA). These genes should theoretically be expressed at a constant level among different tissues of an organism, at all stages of development, and their expression levels should also remain relatively constant in different experimental conditions.

However, none of these housekeeping genes are ideal. It has been shown that *GAPDH* expression levels are altered by glucose, insulin, heat shock, and cellular proliferation; and β-actin levels may also be modified by experimental treatments.[31–35] rRNA production is less likely to vary under conditions affecting mRNA transcription.[36,37] However, it is not always a good representative of total mRNA population in a cell as rRNA is expressed at a much higher level than mRNA.

Other alternative housekeeping genes have been proposed, but none have been entirely satisfactory, and no single unequivocal reference gene has yet been identified. Some authors have suggested the use of several housekeeping genes in a single experiment and that the mean expression of these multiple housekeeping genes can be used for normalization.[38] Importantly, selection of the housekeeping gene for each specific experiment should be made very carefully as the reliability of the results depends on the choice of the most relevant housekeeping gene according to the cells of interest and specific experimental treatments.

AMPLICON DETECTION STRATEGIES

Two general chemistries are available. These include double-stranded (ds) DNA-intercalating agents (DNA-binding dyes) and fluorescent probes. The former includes SYBR Green I or ethidium bromide and is the simplest and most cost-effective method, as amplicon-specific labeled hybridization probes are not required. SYBR Green I only fluoresces when intercalated into

dsDNA. The intensity of the fluorescence signal is therefore dependent on the quantity of dsDNA present in the reaction. The main disadvantage of this method is that it is not specific, because the dye binds to all dsDNAs formed during the PCR reaction (i.e., nonspecific PCR products and primer-dimers).

With fluorogenic probes, nonspecific amplification due to mispriming or primer-dimer artifact does not generate signal, as specific hybridization between probe and template is necessary for fluorescence emission. Also, fluorogenic probes can be labeled with different and distinguishable reporter dyes, thus allowing the detection of amplicons that may have been produced by one or several primer pairs in a single PCR reaction—termed multiplex real-time PCR. However, different probes must be developed to detect different sequences. The various chemistries are now described in more detail.

Double-stranded DNA-intercalating agents (DNA-binding dyes): SYBR Green I is a nonsequence-specific fluorogenic minor groove DNA-binding dye that intercalates into dsDNA (it does not bind to single-stranded DNA). SYBR Green 1 exhibits little fluorescence when unbound in solution but emits a strong fluorescent signal upon binding to dsDNA.[39] An increase in the fluorescence signal occurs during polymerization, and this decreases when DNA is denatured. Fluorescent measurements are performed at the end of the elongation step of each PCR cycle to monitor the increasing amount of amplified DNA. The advantage of this technique is that it is relatively cheap, as it can be used with any pair of primers for any target. However, as the presence of any dsDNA generates fluorescence, specificity of this assay is greatly decreased due to amplification of nonspecific PCR products and primer-dimers.[40] Generating and comparing melting curves (plotting fluorescence as a function of temperature) using the LightCycler™ (Roche Molecular Diagnostics) (or RotorGene, Smart Cycler, iCycler, Mx4000) is one method of increasing the specificity of the reaction.[40] A characteristic melting peak at the melting temperature™ of the amplicon will distinguish it from amplification artifacts that melt at lower temperatures at broader peaks. It is possible to set the software to acquire fluorescence above the primer-dimers' melting temperature but below that of the target. Another controllable problem is that longer amplicons create a stronger signal. Usually, SYBR Green is used in singleplex reactions; however, when coupled with melting-point analysis, it can be used for multiplex reactions. The SYBR Green I reaction has been used for many applications (e.g., viral load detection[41] and cytokine quantifaction.[42–44]

Hydrolysis probes (e.g., TaqMan probes): This chemistry has already been outlined earlier in this review (Figure 8.1). A forward and reverse primer and a probe are used. The efficiency of the assay is mainly dependent on 5′ to 3′ nuclease activity; the most commonly used enzyme is Taq polymerase,[20] but any enzyme with 5′ nuclease activity can be used.[45] The oligonucleotide probe has a covalently bonded fluorescent reporter dye and quencher dye at the 5′ and 3′ ends, respectively. Various fluorescent reporter dyes are in use including 6-carboxyfluorescein (FAM), tetrachloro-6-carboxyfluorescein (TET), hexacholoro-6-carboxyfluorescein (HEX), or VIC. Quenchers include either 6-carboxytetramethylrhodamine (TAMRA) or 4-(dimethylamino-azo) benzene-4-carboxylic acid (DABCYL). When the probe is intact, the proximity of the reporter and quencher dyes permits FRET, and fluorescence emission does not occur. During PCR amplification, the probe anneals to the target, and Taq polymerase cleaves the probe, allowing an increase in fluorescence emission. The increase in fluorescence intensity is directly proportional to the amount of amplicon produced. The TaqMan chemistry is the most widely used real-time PCR assay and has been used for multiple purposes.[32,46,47]

TaqMan minor groove-binding probes have more recently been developed. In this chemistry, the standard TAMRA quencher at the 3′ end is replaced by a nonfluorescent quencher, and a minor groove-binder molecule is also incorporated at the 3′ terminus. The latter stabilizes the probe-target complex by folding into the minor groove of the dsDNA. Additionally, the Tm of the probes is increased, allowing the use of very short oligoprobes (14 nucleotides in length) and providing more accurate allelic discrimination. Thus, TaqMan minor groove-binding probes are ideal for detecting single nucleotide polymorphisms[48,49] and for the quantitative analysis of methylated alleles.[50]

Dual hybridization probes: This method has been convincingly validated in studies using the LightCycler instrument. Two hybridization probes are used—one carries a donor fluorophore at its 3′ end, and the other is labeled with an acceptor fluorophore at its 5′ end. After the denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement during the annealing step. This brings the two dyes in close proximity, allowing FRET. The donor dye in one of the probes transfers energy, allowing the other one to dissipate fluorescence at a different wavelength. The measured fluorescence is directly proportional to the amount of DNA synthesized during the PCR reaction. The specificity of this reaction is therefore increased as a fluo-

rescent signal is only detected when two independent probes hybridize to their correct target sequence. This method has been widely used for detection of minimal residual disease after therapy[51,52] and viral load quantification.[53,54]

Molecular beacons: Molecular beacons also contain covalently bound fluorescent and quenching dyes at either end of a single-stranded DNA molecule. However, they are also designed to adopt a hairpin or stem-and-loop structure while free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur.[55] The loop portion of the molecule is complementary to the target nucleic acid molecule, and the stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. The close proximity of the fluorophore and the quencher in this hairpin configuration suppresses reporter fluorescence. When the probe sequence in the loop hybridizes to a complementary nucleic acid target sequence during the annealing step, a conformational change occurs that forces the stem apart. This results in a linear structure and thus separation of the fluorophore from the quencher dye (FRET does not occur) and an increase in fluorescence emission. A new hybridization takes place in the annealing step of each cycle, and the intensity of the resultant fluorescence indicates the amount of accumulated amplicon at the end of the previous cycle. Molecular beacons remain intact during PCR, and they must rehybridize to the target sequence each cycle for fluorescence emission. Molecular beacons are especially suitable for identifying point mutations.[56–58]

Scorpions: Similar to molecular beacons, scorpions adopt a stem-and-loop configuration with a 5′ fluorophore and 3′ quencher. The specific probe sequence is held within the hairpin loop, which is attached to the 5′ terminus of a PCR primer sequence by a nonamplifiable monomer (termed the PCR stopper). This chemical modification prevents PCR from copying the stem-loop sequence of the scorpion primer. During PCR, scorpion primers are extended to form amplicon. In the annealing phase, the specific probe sequence in the scorpion tail curls back to hybridize to the complementary target sequence in the amplicon, thus opening up the hairpin loop. This prevents the fluorescence from being quenched, and a signal is observed.[59] As the tail of the scorpion and the amplicon are now part of the same strand of DNA, the interaction is intramolecular.

The benefits of scorpions derive from the fact that the probe element is physically coupled to the primer element, which means that the reaction leading to signal generation is a

unimolecular event. This contrasts to the bimolecular collisions required by other technologies such as TaqMan or molecular beacons. The benefits of a unimolecular rearrangement are significant in that the reaction is effectively instantaneous and the fluorescence signal much stronger. Also better discrimination and specificity are achieved using scorpions. Scorpion probes have been used for viral load and mutation detection.[60,61]

Duplex scorpions are a modification of scorpions. However, in contrast to scorpions (or molecular beacons), the fluorophore and quencher dye is separated onto different and complementary oligonucleotides. The advantage of duplex scorpions is the significantly greater separation between the quencher and reporter fluorophore, which decreases fluorophore quenching when the probe is bound to the target, resulting in better signal intensity compared with conventional scorpions.[62]

DESIGNING A PRIMER, PROBE, AND AMPLICON

Great care should go into the design of an assay. Primers, probes, and amplicons are designed to very exacting specifications, and the TaqMan system provides its own primer/probe design software from Applied Biosystems known as Primer Express, which is one of the most widely used oligonucleotide design programs for developing real-time quantitative PCR assays. Primer3, a free program from Massachusetts Institute of Technology (MA, USA), can also be used to generate good real-time PCR assays, including designs incorporating an internal hybridization probe. The amplicon for the PCR product should be as small as reasonably possible, usually 50–150 bp in length for designs using hybridization probes (and less than 300 bp for SYBR Green assays). Shorter amplicons amplify more efficiently and are more tolerant of reaction conditions. The optimal length for single-stranded primers is 15–20 bases with a G/C content of 20%–80%. Their Tm should be in the range of 68°C–70°C for TaqMan primers. Molecular beacon and hybridization probe-associated primers can have a wider range of Tm, but the Tm of any one pair should be similar (i.e., not differ by more than 1°C–2°C). Nonspecific priming is minimized by selecting primers that have only one or two G/Cs within the last five nucleotides at the 3′ end. If using a SYBR Green I approach, the PCR primers must not form an appreciable amount of primer-dimer bands. A melting curve analysis of each product is needed to ensure that the fluorescent signal observed is from the desired PCR product. In mRNA expression assays using a hybridization probe, the probe sequence should span an exon/exon boundary if possible. Having the probe Tm 8°C–10°C

higher than that of the primers ensures that the probe is fully hybridized during primer extension. TaqMan probes should not contain a G at their 5′ ends due to the quenching effect of a G in this position on reporter fluorescence, even after probe cleavage.

PUSHING THE TECHNOLOGY FURTHER: MULTIPLEX QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

The term multiplex Q-RT-PCR is used to describe the use of multiple fluorogenic probes for the discrimination of multiple amplicons in a single tube. The main advantages of multiplexing over single-target analysis are the ability to provide internal controls, lower reagent costs and preservation of precious low quantity samples. The main restrictions of this technique have been the limited number of available fluorophores, fluorescence emission from quenching dyes, and the common use in real-time instruments of a monochromatic light source. The introduction of nonfluorescent quenchers, which have no inherent fluorescence, has been a breakthrough that has allowed an increase in the number of spectrally discernable fluorogenic probes used per reaction. Initial Q-RT-PCR instrumentation contained optimized filters to minimize overlap of the emission spectra from the fluorophores. Newer systems have used either multiple light-emitting diodes, which span the whole visible spectrum, or a tungsten lamp, which emits light over a broad range of wavelengths. However, despite these advancements, only four-color multiplex reactions are usually possible,[63,64] of which one color may be used for an internal control. One recent development is the introduction of combinatorial fluorescence energy transfer tags,[65,66] which will help to boost the development of multiplex real-time PCR.

EQUIPMENT REVIEW

There are a variety of instruments available on the market, each of which has its own individual characteristics. Great care should be taken when choosing which instrument to buy, and it is important to match the instruments capabilities with laboratory needs. Cost should not be the only factor when making a choice; the cheaper models cannot compensate for the variance in the optics and therefore are not capable of detecting smaller differences. The higher-throughput instrument may be more than is needed. The ABI Prism® 7700 Sequence Detection System (SDS) from Applied Biosystems was the first commercially available thermocycler for real-time PCR, but has now been discontinued.

Continuous fluorescence wavelength laser light detection from 500–660 nm allowed multiplex PCR on this machine.

The ABI Prism 7700 has more recently been replaced by the ABI Prism 7900HT, which has similar specifications to the 7700 SDS but is completely automated and designed especially for very high-throughput applications (384 samples per run). Another recent introduction is the less expensive ABI Prism 7000 SDS. It retains the Peltier-based 96-well block thermal cycling format of the ABI 7700, but replaces the laser with a tungsten–halogen lamp that simultaneously illuminates all sample wells. The software supplied with the instrument is much more user friendly and is Microsoft Windows–based, which allows easy export of data and amplification plots. One of the major advantages of the ABI instruments is the collection of data from a passive reference signal to normalize each reaction for variances in the optics of the system. In addition, Applied Biosystems have launched the Applied Biosystems 7300 and 7500 Real Time PCR systems, which represent less expensive alternatives.

The low-priced LightCycler from Roche Molecular Biochemicals induces fluorescence excitation by a blue light-emitting diode that is read by three silicon photodiodes with different-wavelength filters, allowing detection of spectrally distinct fluorophores. Therefore, multiplex PCR can be performed. A complete PCR run of 30–40 cycles is performed in 20–30 min, but only a limited number of samples (maximum 32) can be analyzed simultaneously. As the LightCycler analyzes the specificity of the results by performing melting curves, it makes the use of dsDNA-binding dyes such as SYBR Green I more reliable. However, as samples need to be in capillaries rather than tubes, it is less practical for the investigator.

The iCycler iQ from BioRad Instruments has a tungsten-halogen lamp allowing excitation of a wide range of fluorophores (400–700 nm). It is able to multiplex four different fluorophores per sample tube. Also, it has an optical module, allowing fluorescence emission to be viewed during the course of PCR amplification. Furthermore, the 96 samples are tracked simultaneously, thereby providing a fast assay. A recently launched module allows it to amplify 384 samples at any one time.

A new option is the Mx4000® Multiplex from Stratagene. This sequence detector instrument is able to detect multiple fluorescence PCR chemistries, including TaqMan and hybridization probes, and molecular beacons. The light source for the Mx4000 system is a quartz tungsten–halogen lamp that generates a broad excitation range of 350–750 nm, and there are four photomulti-

plier tubes with a detection range of 350–830 nm. The instrument is ideal for performing multiplex PCR. Importantly, the system contains an integrated personal computer that operates independently from the instrument's embedded microprocessor, which gives some protection against data loss.

The Smart Cycler System has recently become available from Cepheid. The system can be operated with molecular beacons, scorpions, hybridization probes, TaqMan probes, or SYBR Green I. An advantage of this system is its high flexibility, as it contains 16 different modules. Each module can be individually programmed and has its own optical subsystem, and can detect four different fluorophores in one reaction. Different operators can define the parameters for each reaction and different runs can be carried out at the same time for individual experiments. A disadvantage of the basic system is the small sample number (maximum 16); however, this can now be increased to 96 wells per run.

The Rotor Gene™ 3000, designed by Corbett Research, is a centrifugal thermal cycler comparable with the LightCycler. It uses four separate light-emitting diode light sources that excite at 470, 530, 585 and 625 nm. Excitation is detected using six filters and photomultipliers at 510, 555, 610, 660, 580, and 610 nm. The design of this instrument is radically different from all other instruments: the real-time reactions are carried out in standard microfuge tubes inside a 36- or 72-well rotor that spins at 500 rpm. This is meant to remove any temperature equilibration time and nonuniformity, and sample-to-sample variation of less than 0.01°C is claimed.

CONCLUSION

The introduction of real-time PCR technology has revolutionized the field of molecular diagnostics and has enabled the shift of molecular diagnostics toward a high-throughput, automated with lower turnaround times. It allows the sensitive, specific, and reproducible quantification of mRNA. Real-time PCR assays are characterized by a wide dynamic range of quantification of 7–8 logarithmic decades, a high technical sensitivity (<5 copies) and a high precision (<2% standard deviation).[32] Also, no post-PCR steps are required, thus avoiding the possibility of cross contamination due to PCR products. The disadvantages of real-time quantitative PCR when compared with conventional PCR include the fact that:

- Amplicon size cannot be monitored without opening the system
- The limited multiplex capabilities of existing instruments

- The incompatibility of several systems with some fluorogenic chemistries

Real-time PCR technology is only as reliable as the accompanying controls and associated quality assurance programs. This includes the quality of standards and choice of housekeeping gene (the search for the ideal housekeeping gene or protocol is ongoing), the use of suitably controlled standard curves and the need to fully optimize, validate, and evaluate each and every new assay against previously standardized assays. Without such care, real-time PCR will provide an enormous amount of fast but inaccurate data.

The contemporary competition for Q-RT-PCR technology is microarray. However, due to current microarray technologies requiring a large amount of starting material and displaying a limited dynamic range for quantification expression levels of selected genes, true quantification experiments will continue to be conducted using real-time PCR methods.[67,68] Therefore, a combination of both technologies, in which the screening of the involved genes is performed by microarrays and the precise quantification and high throughput screening is performed by real-time PCR, is the ideal method. Similarly, real-time PCR technology will continue to be combined with advanced microdissection techniques[13,16–19] or nucleic acids obtained from paraffin-fixed archival samples.[14,15] The detection and analysis of minimal residual disease[51,69] and viral loads will remain an important application. Also, it will be possible to measure gene expression or DNA copy number in specific cells that are isolated with difficulty and are present in only very small numbers. Combining techniques for sorting fetal cells or DNA from the maternal circulation with real-time PCR will enable early prenatal diagnostics of numerous congenital disorders using minimally invasive procedures.[70–72] Real-time techniques will inevitably be used in the analysis of clinical samples to aid clinicians in prognosis and management of patients with a variety of diverse disease states. Limiting this application at present is the lack of universal agreement on basic issues such as quality and quantity control of RNA, storage standards, guidelines for analysis and reporting of results, and standardization of protocols. These assays are likely to be increasingly utilized as an important area of molecular diagnostics in the future.

References

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

2. Kariyazono H, Ohno T, Ihara K, et al. Rapid detection of the 22q11.2 deletion with quantitative real-time PCR. *Mol Cell Probes* 2001; 15:71–73.
3. Nigro JM, Takahashi MA, Ginzinger DG, et al. Detection of 1p and 19q loss in oligodendroglioma by quantitative microsatellite analysis, a real-time quantitative PCR assay. *Am J Pathol* 2001;4: 1253–1262.
4. Ginzinger DG, Godfrey TE, Nigro J, et al. Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. *Cancer Res* 2000;60:5405–5409.
5. Ingham DJ. The study of transgene copy number and organization. *Methods Mol Biol* 2005;286:273–290.
6. Bai RK, Perng CL, Hsu CH, Wong LJ. Quantitative PCR analysis of mitochondrial DNA content in patients with mitochondrial disease. *Ann NY Acad Sci* 2004;1011:304–309.
7. Desire N, Dehee A, Schneider V, et al. Quantification of human immunodeficiency virus type 1 proviral load by a TaqMan real-time PCR assay. *J Clin Microbiol* 2001;39:1303.
8. Johnson VJ, Yucesoy B, Luster MI. Genotyping of single nucleotide polymorphisms in cytokine genes using real-time PCR allelic discrimination technology. *Cytokine* 2004;27:135–141.
9. Petersen K, Vogel U, Rockenbauer E, et al. Short PNA molecular beacons for real-time PCR allelic discrimination of single nucleotide polymorphisms. *Mol Cell Probes* 2004;18:117–122.
10. Elson D, Thurston G, Huang E, et al. Quiescent angiogenesis in transgenic mice expressing constitutively active hypoxiainducible factor-1a. *Genes Dev* 2001;15:2520.
11. Schmittgen TD, Teske S, Vessella RL, True LD, Zakrajsek BA. Expression of prostate specific membrane antigen and three alternatively spliced variants of PSMA in prostate cancer patients. *Int J Cancer* 2003;107:323–329.
12. Caberlotto L, Hurd YL, Murdock P, et al. Neurokinin 1 receptor and relative abundance of the short and long isoforms in the human brain. *Eur J Neurosci* 2003;17:1736–1746.
13. Sethi N, Palefsky J. Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection. *FASEB J* 2004;18:1243–1245.
14. Godfrey TE, Kim SH, Chavira M, et al. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5′ nuclease quantitative reverse transcription-polymerase chain reaction. *J Mol Diagn* 2000;2:84–91.
15. Andreassen CN, Sorensen FB, Overgaard J, Alsner J. Optimization and validation of methods to assess single nucleotide polymorphisms (SNPs) in archival histological material. *Radiother Oncol* 2004;72: 351–356.
16. Glockner S, Lehmann U, Wilke N, Kleeberger W, Langer F, Kreipe H. Detection of gene amplification in intraductal and infiltrating breast cancer by laser-assisted microdissection and quantitative real-time PCR. *Pathobiology* 2000;68:173–179.

17. Ehrig T, Abdulkadir SA, Dintzis SM, Milbrandt J, Watson MA. Quantitative amplification of genomic DNA from histological tissue sections after staining with nuclear dyes and laser capture microdissection. *J Mol Diagn* 2001;3:22–25.
18. Fink L, Seeger W, Ermert L, et al. Real-time quantitative RTPCR after laser-assisted cell picking. *Nat Med* 1998;4:1329–1333.
19. Shieh DB, Chou WP, Wei YH, Wong TY, Jin YT. Mitochondrial DNA 4,977-bp deletion in paired oral cancer and precancerous lesions revealed by laser microdissection and real-time quantitative PCR. *Ann NY Acad Sci* 2004;1011:154.
20. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci USA* 1991;88:7276–7280.
21. Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res* 1993;21:3761–3766.
22. Cardullo RA, Agrawal S, Flores C, Zamecnick PC, Wolf DE. Detection of nucleic acid hybridization by non-radiative fluorescence resonance energy transfer. *Proc Natl Acad Sci USA* 1988;85:8790.
23. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986–994.
24. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Res* 1996;6:995–1001.
25. Dumur CI, Dechsukhum C, Wilkinson DS, Garrett CT, Ware JL, Ferreira-Gonzalez A. Analytical validation of a real-time reverse transcriptionpolymerase chain reaction quantitation of different transcripts of the Wilms' tumor suppressor gene (WT1). *Anal Biochem* 2002;309:127–136.
26. Jurado J, Prieto-Alamo MJ, Madrid-Risquez J, Pueyo C. Absolute gene expression patterns of thioredoxin and glutaredoxin redox systems in mouse. *J Biol Chem* 2003;278:45546.
27. Borg I, Rohde G, Loseke S, et al. Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. *Eur Respir J* 2003;21:944–951.
28. Lin JC, Wang WY, Chen KY, et al. Quantification of plasma Epstein-Barr virus DNA in patients with advanced nasopharyngeal carcinoma. *N Engl J Med* 2004;350:2461–2470.
29. Castelain S, Descamps V, Thibault V, et al. TaqMan amplification system with an internal positive control for HCV RNA quantitation. *J Clin Virol* 2004;31:227–234.
30. Gilliland G, Perrin S, Bunn HF. Competitive PCR for quantitation of mRNA. In: PCR Protocols: A Guide to Methods and Applications. Innis, MA, ed. CA, USA: Academic Press, 1990, 60–69.
31. Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. *BioTechniques* 2000;29:332–337.
32. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169–193.

33. Rhoads RP, McManaman C, Ingvartsen KL, Boisclair YR. The housekeeping genes GAPDH and cyclophilin are regulated by metabolic state in the liver of dairy cows. *J Dairy Sci* 2004;87:248.
34. Steele BK, Meyers C, Ozbun MA. Variable expression of some "housekeeping" genes during human keratinocyte differentiation. *Anal Biochem* 2002;307:341–347.
35. Yperman J, De Visscher G, Holvoet P, Flameng W. β-actin cannot be used as a control for gene expression in ovine interstitial cells derived from heart valves. *J Heart Valve Dis* 2004;13:848.
36. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *BioTechniques* 2004;37:112, 116, 118.
37. BasA, Forsberg G, Hammarstrom S, Hammarstrom ML. Utility of the housekeeping genes 18S rRNA, β-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand J Immunol* 2004;59:566–573.
38. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:0034.I.
39. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *BioTechniques* 1998;24:954–958, 960, 962.
40. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem* 1997;245:154–160.
41. Gibellini D, Vitone F, Schiavone P, Ponti C, La Placa M, Re MC. Quantitative detection of human immunodeficiency virus type 1 (HIV-1) proviral DNA in peripheral blood mononuclear cells by SYBR green real-time PCR technique. *J Clin Virol* 2004;29:282–289.
42. Blaschke V, Reich K, Blaschke S, Zipprich S, Neumann CJ. 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. *Immunol Methods* 2000;246:79–90.
43. Ramos-Payan R, Aguilar-Medina M, Estrada-Parra S, et al. Quantification of cytokine gene expression using an economical real-time polymerase chain reaction method based on SYBR Green I. *Scand J Immunol* 2003;57:439–445.
44. Nakamura T, Scorilas A, Stephan C, et al. Quantitative analysis of macrophage inhibitory cytokine-1 (MIC-1) gene expression in human prostatic tissues. *Br J Cancer* 2003;88:1101–1104.
45. Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H. One-tube fluorogenic reverse transcriptionpolymerase chain reaction for the quantitation of feline coronaviruses. *J Virol Methods* 1999;77:37–46.

46. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 2001;25:386–401.
47. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 2002;30:503–512.
48. van Hoeyveld E, Houtmeyers F, Massonet C, et al. Detection of single nucleotide polymorphisms in the mannosebinding lectin gene using minor groove binder-DNA probes. *J Immunol Methods* 2004;287: 227–230.
49. de Kok JB, Wiegerinck ET, Giesendorf BA, Swinkels DW. Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). *Hum Mutat* 2002;19:554–559.
50. Zeschnigk M, Bohringer S, Price EA, Onadim Z, Masshofer L, Lohmann DR. A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. *Nucleic Acids Res* 2004;32:E125.
51. Emig M, Saussele S, Wittor H, et al. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. *Leukemia* 1999;13:1825–1832.
52. van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003;17: 1013–1034.
53. Schalasta G, Eggers M, Schmid M, Enders G. Analysis of human cytomegalovirus DNA in urines of newborns and infants by means of a new ultrarapid real-time PCR-system. *J Clin Virol* 2000;19: 175–185.
54. Aliyu SH, Aliyu MH, Salihu HM, Parmar S, Jalal H, Curran MD. Rapid detection and quantitation of hepatitis B virus DNA by real-time PCR using a new fluorescent (FRET) detection system. *J Clin Virol* 2004;30:191–194.
55. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnol* 1996;14:303–308.
56. Smit ML, Giesendorf BA, Vet JA, Trijbels FJ, Blom HJ. Semiautomated DNA mutation analysis using a robotic workstation and molecular beacons. *Clin Chem* 2001;47;739–744.
57. Abravaya K, Huff J, Marshall R, et al. Molecular beacons as diagnostic tools: technology and applications. *Clin Chem Lab Med* 2003;41: 468–474.
58. Wabuyele MB, Farquar H, Stryjewski W, et al. Approaching real-time molecular diagnostics: single-pair fluorescence resonance energy transfer (spFRET) detection for the analysis of low abundant point mutations in K-ras oncogenes. *J Am Chem Soc* 2003;125:6937–6945.

59. Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and flourescence. *Nature* 1999;17:804.
60. Hart KW, Williams OM, Thelwell N, et al. Novel method for detection, typing, and quantification of human papillomaviruses in clinical samples. *J Clin Microbiol* 2001;39:3204–3212.
61. Thelwell N, Millington S, Solinas A, Booth J, Brown T. Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res* 2000;28:3752–3761.
62. Solinas A, Brown LJ, McKeen C, et al. Duplex Scorpion primers in SNP analysis and FRET applications. *Nucleic Acids Res* 2001;29: E96.
63. Ugozzoli LA, Hamby K. Four-color multiplex 5′ nuclease assay for the simultaneous detection of the factor V Leiden and the prothrombin G20210A mutations. *Mol Cell Probes* 2004;18:161–166.
64. Vet JA, Majithia AR, Marras SA, et al. Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc Natl Acad Sci USA* 1999;96:6394–6399.
65. Tong AK, Li Z, Jones GS, Russo JJ, Ju J. Combinatorial fluorescence energy transfer tags for multiplex biological assays. *Nature Biotechnol* 2001;19:756–759.
66. Tong AK, Ju J. Single nucleotide polymorphism detection by combinatorial fluorescence energy transfer tags and biotinylated dideoxynucleotides. *Nucleic Acids Res* 2002;30:E19.
67. Rickman D, Bobek MP, Misek DE, et al. Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. *Cancer Res* 2001;65:6885–6891.
68. Miyazato A, Ueno S, Ohmine K, et al. Identification of myelodysplastic syndrome-specific genes by DNA microarray analysis with purified hematopoietic stem cell fraction. *Blood* 2001;98:422–427.
69. Dolken G. Detection of minimal residual disease. *Adv Cancer Res* 2001;82:133.
70. Lo YM, Tein MS, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998:62:768–775.
71. Hu Y, Zheng M, Xu Z, Wang X, Cui H. Quantitative real-time PCR technique for rapid prenatal diagnosis of Down syndrome. *Prenat Diagn* 2004;24:704–707.
72. Costa C, Pissard S, Girodon E, Huot D, Goossens M. A one-step real-time PCR assay for rapid prenatal diagnosis of sickle cell disease and detection of maternal contamination. *Mol Diagn* 2003;7:45–48.
73. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR. Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn* 2005;5:209–219.