Twenty-Five Years of Quantitative PCR for Gene Expression Analysis

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Following its invention 25 years ago, PCR has been adapted for numerous molecular biology applications. Gene expression analysis by reverse-transcription quantitative PCR (RT-qPCR) has been a key enabling technology of the post-genome era. Since the founding of BioTechniques*, this journal has been a resource for the improvements in qPCR technology, experimental design, and data analysis. qPCR and, more specifically, real-time qPCR has become a routine and robust approach for measuring the expression of genes of interest, validating microarray experiments, and monitoring biomarkers. The use of real-time qPCR has nearly supplanted other approaches (e.g., Northern blotting, RNase protection assays). This review examines the current state of qPCR for gene expression analysis now that the method has reached a mature stage of development and implementation. Specifically, the different fluorescent reporter technologies of real-time qPCR are discussed as well as the selection of endogenous controls. The conceptual framework for data analysis methods is also presented to demystify these analysis techniques. The future of qPCR remains bright as the technology becomes more rapid, cost-effective, easier to use, and capable of higher throughput.*

INTRODUCTION

BioTechniques began publication in 1983, the same year as the discovery of PCR by Kary Mullis (1). Both PCR and *BioTechniques* have enjoyed an exponential rise in popularity in the ensuing 25 years. Although quantitative PCR (qPCR) was originally developed for, and is still used for, DNA quantitation (e.g. viral load), this review focuses on the use of qPCR for quantitation of RNA levels (2,3). A number of early qPCR methods relying on end point analysis of PCR products were proposed soon after its development (4,5,6). qPCR came of age, however, with the introduction of real-time qPCR methods. It is now more than a decade since the initial publications describing real-time quantitative PCR (7,8,9,10). As can be seen in Figure 1, citations of qPCR increased dramatically (following a brief lag during adoption of the technology) with the introduction of real-time qPCR. In fact, the level of citations from qPCR is reaching the end of the exponential growth phase (not dissimilar from a real-time amplification plot).

Throughout the past 25 years, *BioTechniques* has been an important source of information on qPCR. The initial report of qPCR methods in *BioTechniques* in 1992 (11) has been followed by more than 80 additional reports in the journal. Additionally, two major reviews of the qPCR method have been published in *BioTechniques,* including one from Zimmermann and Mannhalter in 1996 (12) and a contribution by us in 1999 (13). In this review, we will assess the changes in the qPCR field for gene expression analysis in the past nine years and describe the maturation of this technology from optimization to standard use. We will focus on the use of qPCR to measure levels of RNA species and specifically try to demystify a recurrent confusion in the field: mathematical analysis of qPCR data. There are numerous applications for qPCR in the genetics field (e.g., gene deletion, gene duplication (14)), but we will not address these in the present context. Instead, our focus is on the ability of qPCR—more specifically reverse-transcription quantitative PCR (RT-qPCR)—to measure the levels of mRNAs, miRNAs, and other RNA species. This is obviously of great significance to modern biology and biomedical sciences. In fact, qPCR has progressed in tandem with the microarray field, which we have reviewed previously in *BioTechniques* (15).

qPCR now represents the method of choice for analyzing gene expression of a moderate number of genes in anywhere from a small number to thousands of samples. For investigators studying gene expression, there is a multitiered technological approach depending on the number of genes and samples being examined. Gene expression microarrays are still the preferred method for large-scale (e.g., whole-genome) discovery experiments (Figure 2). Due to the logistics, sensitivity, and costs of whole-genome microarrays, there is also a niche for focused microarrays that allow for analysis of a smaller number of genes in a larger number of samples. Nonetheless, for validation of microarray discovery, RT-qPCR remains the gold standard (16). The current maturation of real-time qPCR with fluorescent probes allows for rapid and easy confirmation of microarray results in a large number of samples. Often, a whole-genome discovery experiment is not required, as the gene or pathway of interest is already known. In that case, the data collection can begin with qPCR. Finally, qPCR has also shown great utility in biomarker monitoring. In this scenario, previously developed identified targets can be assayed in very large numbers of samples (1000s).

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While we have focused on gene expression, it is important to define that term more specifically. Generally, this refers to mRNA levels. However, since the original discovery of microRNAs (miRNA) (17), thousands of these regulating RNAs have been described (18). In the same manner as mRNAs, miRNA assays have been developed for real-time qPCR (19). The expanding understanding of other RNA species, including small nuclear, small nucleolar, and piwi-interacting RNAs, means that more types of RNAs will be assayed in the future (19).

Technologies

In 1977, the Northern blot was introduced for RNA analysis and named after the DNA Southern blot invented by Sir Edwin Southern (20). Northern blotting uses denaturing gel electrophoresis and blotting with hybridization probe-dependent detection of target RNAs. RNA levels can be quantified and directly compared between multiple samples on a single membrane, although this method lacks the accuracy of fluorescence-based qPCR. The Northern blot is, however, still quite useful in the study of RNA degradation and transcript size, although nonspecific hybridization can confound data interpretation and the use of radioactivity is frequently necessary (21,22). The RNase protection assay is also used to detect, quantify, and characterize RNA species due to its sensitivity and specificity (23,24). Hybridization of antisense RNA corresponding to a known complementary target sequence prevents target digestion by single strand–specific RNase activity. This process results in the degradation of all remaining single-stranded RNAs (i.e., those not hybridized to the probe sequence), enabling the accurate quantitation of specific target sequences. These techniques, while revolutionary at the time of their inception, are long and involved protocols that are not amenable to the examination of many different transcripts in a high-throughput manner. Furthermore, the required amount of RNA can be quite large.

With the advent of PCR, vanishingly small amounts of RNA, converted into cDNA by the reverse-transcription reaction, could be amplified. This amplification, however, poses a challenge for accurately quantifying the initial amount of cDNA. A PCR of only 20 cycles amplifies the initial
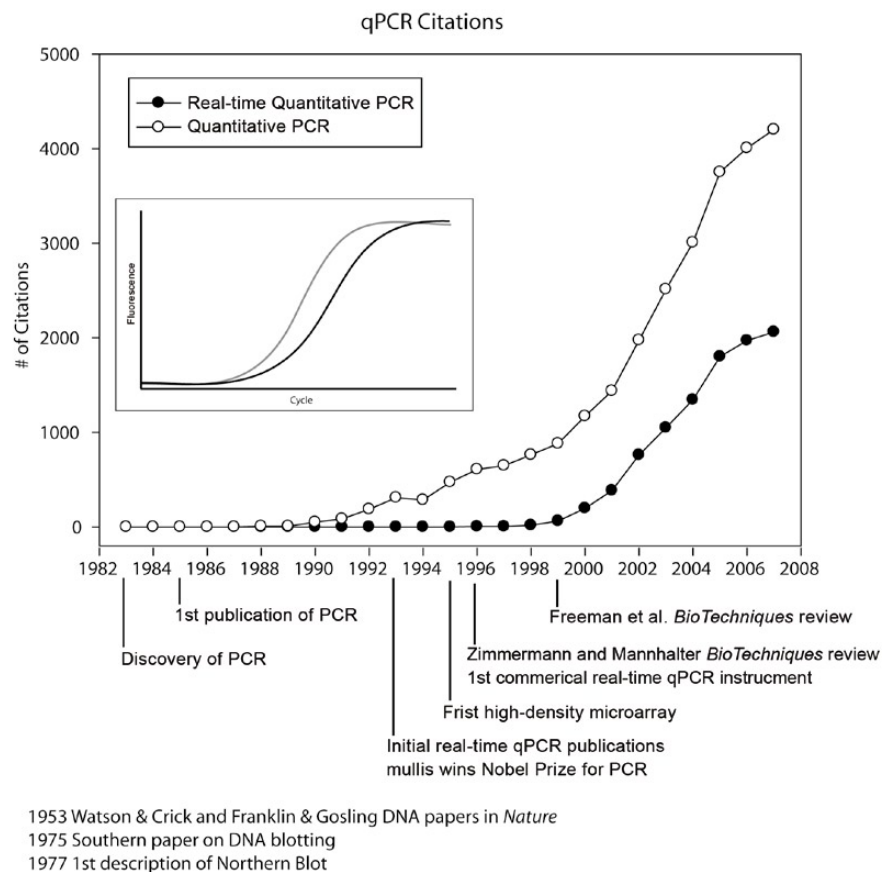

Figure 1. Growth of qPCR as observed through citations. PubMed searches using the keywords "quantitative PCR" (open circles) and "real-time quantitative" PCR (filled circles) were used to gauge the growth of quantitative PCR in the literature. Specific dates of inventions and reviews are given at the bottom of the graph. The growth of publications using these techniques resembles the amplification plot generated in a PCR (inset).

cDNA over a million-fold (assuming 100% reaction efficiency). With this tremendous gain there is also the potential for huge errors. For example, a 95% efficient PCR will only amplify the original cDNA over 600,000-fold. Accurate qPCR quantitation therefore requires that all samples have equal amplification efficiency.

RT-qPCR was initially developed as a technique to amplify distinct nucleic acid sequences to allow for detection and relative quantification of target RNAs (6). Real-time qPCR technology has improved upon RT-qPCR by facilitating the simultaneous amplification and quantitation of specific nucleic acid sequences throughout the course of the PCR (25). Some confusion in nomenclature has arisen from the use of RT-qPCR to refer also to real-time qPCR. In this review, RT-qPCR refers only to reverse-transcription qPCR. The current level of qPCR development and implementation enables routine and precise quantitation of gene expression levels. A full range of technologies is now

available for real-time qPCR whether examining 1 gene in 20 samples or 20 genes in 200 samples.

End point qPCR, while the initial technology for quantitation, has some major limitations. DNA (either genomic or cDNA derived from RNA) is amplified for a discrete number of cycles and the resulting products are then subjected to analysis. The products must be visualized, generally through agarose gel electrophoresis and staining (e.g., ethidium bromide) (26). This is both time consuming and is not reliably quantitative. The densitometry of the gels is limited in its dynamic range and lacks sensitivity (27). Most importantly, though, the quantitation may be incorrect. Examination of a PCR amplification plot (Figure 3) shows the reaction going through exponential and linear phases in which the PCR is initially not limited by enzymatic activity or substrates. Eventually, the enzyme activity and/or the substrates (e.g., dNTPs) become limiting or exhausted. At this point, the reaction reaches a plateau

and theoretically, all of the samples will reach the same total amount of amplified DNA. This both obscures any difference in initial cDNA abundance and is also quite variable. In practice, the plateau of the reaction for different samples will be at different levels for a variety of reasons. By quantifying PCR products at the end of the reaction after a high number of cycles, any small difference in reaction efficiencies between samples is magnified. For all of these reasons, the use of the end point qPCR technique is in decline. While it is still used, it often is meant to illustrate binary relationships (i.e., expression vs. no expression) such as in knockout studies. The standard for qPCR has become real-time qPCR, which avoids many of the pitfalls of end point qPCR (although it is not without caveats).

While end point qPCR requires that PCR products be detected and quantified by gel electrophoresis after completion of the reaction, real-time qPCR technology allows quantification of PCR products in "real time" during each PCR cycle, yielding a quantitative measurement of PCR products accumulated during the course of the reaction. Real-time reactions are carried out in a thermocycler that permits measurement of a fluorescent detector molecule, which decreases post-processing steps and minimizes experimental error. This is most commonly achieved through the use of fluorescence-based technologies (Table 1), including: (*i*) probe sequences that fluoresce upon hydrolysis (TaqMan; Applied Biosystems, Foster City, CA, USA) or hybridization (LightCycler; Roche, Indianapolis, IN, USA); (*ii*) fluorescent hairpins; or (*iii*) intercalating dyes (SYBR Green). These approaches require less RNA than end point assays, possess a wider dynamic range than gel-based densiometry (five orders of magnitude vs. two orders of magnitude), and are more resistant to nonspecific amplification (9,28).

TaqMan chemistry uses the 5′-3′ exonuclease activity of Taq DNA polymerase, which degrades a nonextendable fluorescent DNA probe following hybridization and extension in the PCR (9). Sequence-specific TaqMan probes are labeled with both a fluorescent reporter and a quencher, which are maintained in close proximity until hybridization to the target occurs. In conventional TaqMan technology, the reporter fluorophore [fluoroscein (FAM)] and fluorescent

Figure 2. **Technologies of measuring gene expression.** Different technological approaches to measuring gene expression depend on the number of samples and the number of genes being examined. As the number of genes decreases and the number of samples increases, the technologies go from being discovery tools to tools for validation and implementation.

quencher [rhodamine (TAMRA)] are bound to the 5′ and 3′ ends of the probe sequence, and fluorescence-resonance energy transfer (FRET) from the reporter to the quencher achieves suppression of reporter molecule fluorescence. Additional reporter/quencher combinations are available, and exhibit differential efficacy based on spectral overlap and experimental design, as is the case with other fluorogenic qPCR technologies (29). Following annealing of the forward and reverse primers to the target sequence, the TaqMan probe is designed to anneal between these primer sites and is hydrolyzed by the 5′-3′ exonuclease activity of the Taq polymerase. If no product is present, the probe does not bind and is not degraded; hence the reporter remains quenched. Probe hydrolysis results in de-suppression of the reporter and a subsequent cumulative increase in fluorescence proportional to the amount of transcript present. This oligonucleotide primer/probe approach increases accuracy and specificity of PCR product detection due to the requirement for precise, gene-specific matching of three independent nucleotide sequences (30). Using a different fluorophore on each gene-specific probe, reactions can be

multiplexed to quantify multiple genes in a single reaction (31,32).

LightCycler technology uses two fluorescent oligonucleotide probes (HybProbes; Roche) (one labeled at the 3′ and the other at the 5′ end) with a phosphorylation modification at the 3′ end to prevent extension. Following target amplification, LightCycler primers hybridize to the target in a head-to-tail configuration, bringing the fluorophores into close proximity. Excitation of the donor fluorophore by an LED light source results in a FRET-based increase in fluorescence emitted by the energy-accepting reporter fluorophore (33,34). Although both TaqMan and LightCycler implement a double-label system and have been reported to be of similar accuracy and performance, LightCycler hybridization probes do not require the probe hydrolysis necessary for fluorescence emission and detection with TaqMan. A dedicated carousel-based LightCycler thermocycling real-time PCR system conducts PCRs in glass capillaries designed to enable rapid air-controlled temperature cycling, thereby decreasing reaction times by approximately 50% (35). Roche recently released a thermal-block LightCycler system that uses 96- and 384-well microplates in
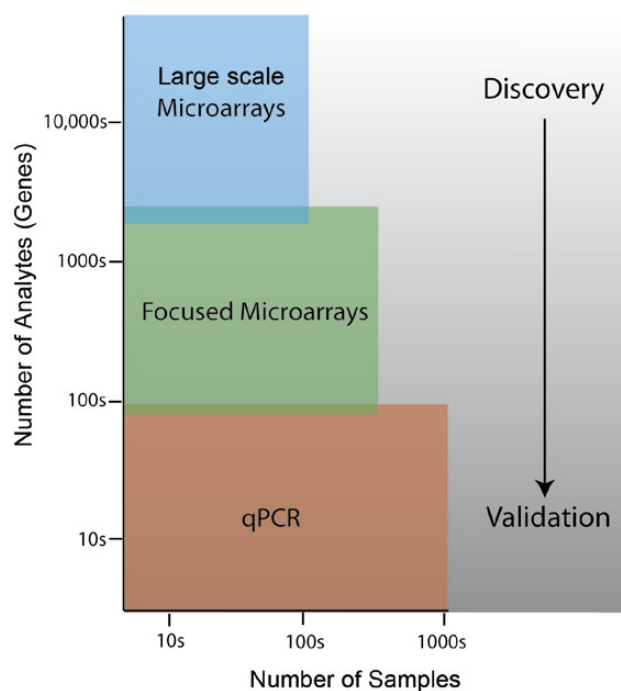
conjunction with multiplexing capabilities to increase throughput.

The LUX (Light Upon eXtension, Invitrogen, Carlsbad, CA, USA) detection technology uses a fluorogenic hairpin primer. When not bound to the target sequence, the 3′ end of the primer interacts with the 5′ end of the primer to provide quenching of the fluorescent moiety near the 3′ end. Hybridization to the target sequence causes an "unfolding" of the hairpin primer, abolishing the quenching effect and resulting in a substantial increase in reporter fluorescence (36). Current LUX technology is highly sensitive and is capable of detecting fewer than 100 copies of target genes. Similar to TaqMan and LightCycler technology, the LUX system allows multiplexed amplification of multiple targets to increase throughput, which is simplified by the single-labeled single-primer approach (37). Melting curve analysis must be implemented with this technology, however, to identify genuine products versus reaction artifacts.

Molecular beacons form stem-loop hairpin structures wherein the loop sequences are complementary to the gene target and the stem sequences are complementary to each other (38). The ends are labeled with a fluorophore and a quencher, respectively, and since molecular beacons adopt a hairpin structure in solution, the reporter is effectively quenched until hybridization to the target sequence occurs. Probe hybridization is highly specific by nature because molecular beacons are designed to thermodynamically favor the quenching hairpin structure rather than to hybridize to nonexact matches (39). Like many others, this approach is compatible with multiplexing, but requires performance evaluation characterization prior to experimentation, due to variations in thermodynamic and hybridization properties, and also requires melting curve analysis for product verification (40).

SYBR Green is an example of an intercalating dye that fluoresces upon binding to double-stranded DNA, much like the workhorse dye, ethidium bromide. Following primer-mediated replication of the target sequence during PCR, multiple molecules of SYBR Green bind to the product and emit a strong fluorescent signal that is easily detected. Intercalating dyes are inexpensive and simple to use compared to sequence probes and, because they are not sequence-specific, they can be used for any reaction. However, because

they do not discriminate between gene sequences, they cannot be used for multiplexed analyses. Further, since SYBR Green binds indiscriminately to double-stranded DNA, primer-dimer artifacts and amplification errors contribute to the detected fluorescence (41). A consequence of multiple dye molecules binding is that the amount of signal is dependent on the mass of double-stranded DNA product created in the reaction. Thus, if the amplification efficiencies are the same, amplification of a longer product will generate more signal than a shorter one would. This is in contrast to the use of a fluorogenic probe, in which a single fluorophore

is released from quenching for each amplified molecule synthesized, regardless of its length.

Fluorescence-based, real-time qPCR technology provides rapid highly sensitive detection of PCR products. Accurate quantitation of reporter fluorescence can be impeded, however, by noise stemming from intrinsic fluorescence of the quencher molecule. The development of "dark quenchers," or nonfluorescent quenching dyes, has circumvented this limitatation (42). Dark quenchers accept fluorescence emitted by reporter dyes without re-emitting it as a detectable signal. These molecules are particularly useful for high-
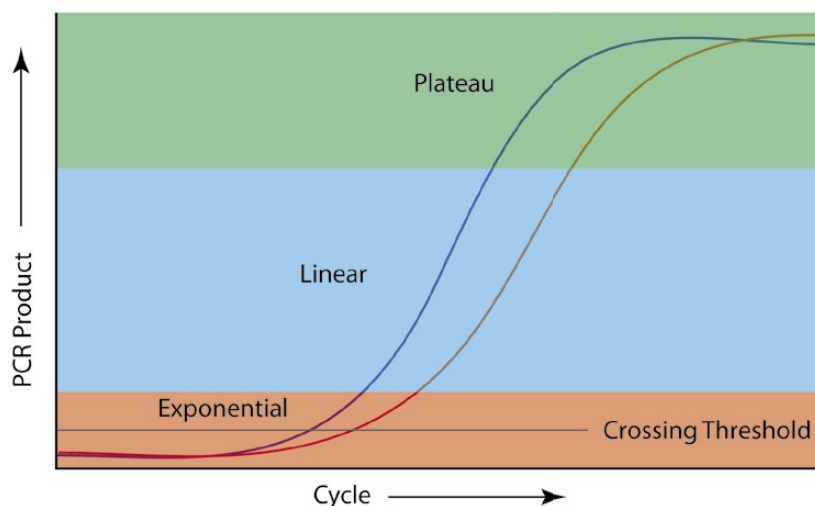

Figure 3. **Phases of PCR.** The PCR process goes through three main phases as the number of cycles and the amount of product generated increase. Initially, when the amount of product is small and enzyme and reagents are not limiting, product generation is exponential and the reaction is closest to 100% efficiency. This exponential growth is hard to detect initially through real-time fluorescence because the amount of product is small. During the linear phase products continue to accumulate, but the reaction efficiency begins to fall and reagents become limiting. Finally, in the plateau phase of the reaction, accumulation of product ceases as the reaction is exhausted for a number of different reasons.

Table 1. Real-time qPCR Technologies

Technology	Supplier	Fluorescent Signal	Multiplexing	Melting Curve Analysis
TaqMan	Applied Biosystems	probe hydrolysis	yes	not necessary
LightCycler	Roche	probe hybridization	yes	not necessary
LUX	Invitrogen	hairpin probe hybridization	yes	necessary
Molecular Beacons	Sigma-Aldrich, IDT	hairpin-loop hybridization	yes	necessary
SYBR Green	Multiple	intercalating dye	no	necessary

A number of fluorophore technologies exist for performing real-time qPCR. Depending on the technology used, the fluorescent signal is generated through different events. Some technologies can be multiplexed and some technologies require melting curve analysis to determine the specificity of the amplification. Specific references for each technology are provided in the text.
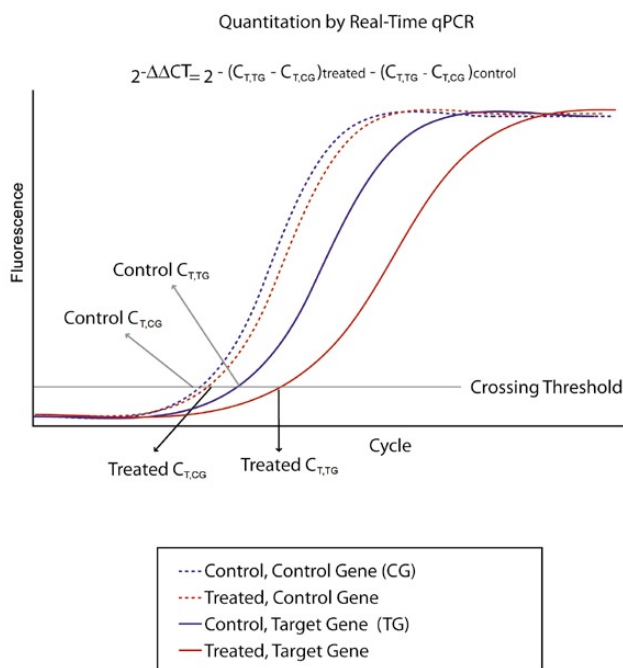
Quantitation by Real-Time qPCR

$$2^{-\Delta\Delta CT} = 2^{-\left[(C_{T,TG} - C_{T,CG})\text{treated} - (C_{T,TG} - C_{T,CG})\text{control}\right]}$$

Figure 4. Mathematical basis of the 2$^{-\Delta\Delta CT}$ method. The 2$^{-\Delta\Delta CT}$ method enables relative quantitation (treated sample is X fold of control sample) through measurements of crossing thresholds (C_T). As described in the text, the comparative differences between the gene of interest and an endogenous control for each sample enable a relative quantitative comparison between the samples. Note that the endogenous control genes (dotted lines) do not vary significantly in C_T with treatment. While this is an assumption of the method, it can be empirically tested by the experimenter.

throughput multiplexed assays because they decrease the number of wavelengths required per reaction and decrease spectral overlap of fluorophores.

Like dark quenchers, minor groove binders (MGBs) offer a simple but effective improvement upon existing RT-qPCR technology. MGB molecules are conjugated to probes to increase the probe melting temperature. This allows greater sensitivity of discrimination between mismatched sequences and increases gene specificity (i.e., detection of single-nucleotide polymorphisms) (43). Additionally, by decreasing the required length of the probe, while increasing the melting temperature, MGB-conjugation facilitates the design of short yet highly specific probe sequences (44).

With the widespread use of qPCR to measure gene expression, alternative methods have been developed that differ significantly from real-time detection methods described above. A competitive qPCR with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) detection has been commercialized by Sequenom (Sequenom, San Diego, CA, USA) (45). This approach

offers the promise of a high-throughput assay. The Quantigene technology (Panomics, Fremont, CA, USA) (46) is a non-PCR–based technology that has been commercialized by Panomics. This technology differs from qPCR in that there is no PCR amplification; rather, capture and amplifier probes are used in conjunction with a chemilumigenic substrate.

Data Analysis

Analysis of real-time qPCR data has also reached a mature stage of development. Analyses can be either of absolute levels (i.e., numbers of copies of a specific RNA per sample) or relative levels (i.e., sample 1 has twice as much mRNA of a specific gene as sample 2). By far, the majority of analyses use relative quantitation as this is easier to measure and is of primary interest to researchers examining disease states. For absolute quantitation, an RNA standard curve of the gene of interest is required in order to calculate the number of copies. In this case, a serial dilution of a known amount (number of copies) of pure RNA is diluted and subjected to amplification. Like a protein assay, the unknown

signal is compared with the curve so as to extrapolate the starting concentration. Alternatively, a computation method for absolute quantitation has been proposed that does not use standard curves (47).

The most common method for relative quantitation is the 2$^{-\Delta\Delta CT}$ method (48). This method relies on two assumptions. The first is that the reaction is occurring with 100% efficiency; in other words, with each cycle of PCR, the amount of product doubles. This can be ascertained through a simple experiment as described in detail by Livak and Schmittgen (48). This assumption is also one of the reasons for using a low cycle number when the reaction is still in the exponential phase. In the initial exponential phase of PCR, substrates are not limiting and there is no degradation of products. In practice, this requires setting the crossing threshold (C_T) at the earliest cycle possible (Figure 3). The C_T is the number of cycles that it takes each reaction to reach an arbitrary amount of fluorescence. The second assumption of the 2$^{-\Delta\Delta CT}$ method is that there is a gene (or genes) that is expressed at a constant level between the samples. This endogenous control will be used to correct for any difference in sample loading. Choice of the endogenous control is important and is discussed in the experimental design section.

Once the C_T value is collected for each reaction, it can be used to generate a relative expression level. The 2$^{-\Delta\Delta CT}$ method is described in Figure 4. In this example, there are two samples (Control and Treated) and we have measured the levels of (*i*) a gene of interest (Target Gene (TG)) and (*ii*) an endogenous control gene (Control Gene (CG)). For each sample, the difference in C_T values for the gene of interest and the endogenous control is calculated (the ΔC_T). Next, subtraction of the control-condition ΔC_T from the treated-condition ΔC_T yields the $\Delta\Delta C_T$. The negative value of this subtraction, the -$\Delta\Delta C_T$, is used as the exponent of 2 in the equation and represents the difference in "corrected" number of cycles to threshold. The exponent conversion comes from the fact that the reaction doubles the amount of product per cycle. For example, if in Figure 4 the control sample ΔC_T is 2 and the treated sample ΔC_T is 4, computing the 2$^{-\Delta\Delta CT}$ (which becomes 2$^{-(4-2)}$) yields 0.25. This value is often referred to as the RQ, or relative quantity value. This means that the level of the gene of interest in the treated sample is only 25% of the level of that

Table 2. Web Resources for Primers and Probes

	Name	Host	URL	# of Primer Sets	Species	Detection
Noncommercial	QPDD	NCI	web.ncifcrf.gov/rtp/gel/primerdb/		Human Mouse	SG, TM
	RTPrimerDB	Ghent University	medgen.ugent.be/rtprimerdb/index.php	3900	Multiple	SG, TM
	Primer Bank	Harvard University	pga.mgh.harvard.edu/primerbank/	306,800 (computed)	Human Mouse	SG
	qPrimerDepot	NCI	primerdepot.nci.nih.gov/		Mouse	SG
Commercial	All Genes	Applied Biosystems	www.allgenes.com	>750,000	Multiple	TM
	GeneGlobe	Qiagen	www1.qiagen.com/GeneGlobe/Default.aspx		Human Mouse Rat	SG
	RT² qPCR Primer	SuperArray	www.superarray.com/RT2PCR.php		Human Mouse Rat	SG
	Real Time Primers	Real Time Primers	www.realtimeprimers.com/	100s	Multiple	SG
	Quantigene	Panomics	www.panomics.com	>1500	Human Mouse Rat	NA

With the widespread use of real-time qPCR, it is possible to make use of primers and probes already developed by other laboratories or by commercial suppliers instead of designing new probes and primers from each gene of interest. SG, SYBR Green, TM, TaqMan.

gene in the control sample. This becomes obvious because the treated sample took two more cycles of PCR to reach the same amount of product as the control sample and therefore there was less of that cDNA to begin with in the treated sample. It should be noted that investigators occasionally use C_T values directly in statistical calculations. This is to be avoided because it is mathematically incorrect. C_T values are an exponent and cannot be used in t-tests or ANOVAs that require a normal distribution (48). The C_T values must be analyzed by the $2^{-\Delta\Delta C_T}$ or some other method that produces a linear relationship for standard statistical tests. The $2^{-\Delta\Delta C_T}$ method is the most common quantitation strategy, but it should be noted that there are other valid methods for analyzing qPCR C_T values. Michael Pfaffl and colleagues (49,50) and others (51) have also proposed alternative analysis methods.

Experimental Design

Due to the exquisite sensitivity of modern PCR and the inevitable occurrence of experimental variation, careful selection of endogenous controls is imperative to fully exploit the quantitative potential of qPCR (52). Intersample variation between biological and technical replicates can interfere with data analysis and therefore must be normalized to one or more endogenous control genes. Properly selected control genes will normalize differences in the amount and quality of starting material as well as in reaction efficiency. Normalization uses endogenous housekeeping, or reference, genes with the assumptions that their expression is: (*i*) similar between all samples in a given study; (*ii*) is resistant to experimental conditions; and (*iii*) undergoes all steps of the qPCR with the same kinetics as the target gene (52). Housekeeping genes such as β-actin, GAPDH, cyclophilin, or tubulin are commonly used since they are ubiquitously expressed in cells and tissues (53,54). Additionally, transcription of these genes is generally resistant to experimental conditions, making them suitable endogenous controls for single-gene normalization (52,55). The use of a single gene for data normalization can decrease cost and increase throughput, and is a standard approach for normalizing qPCR (52,53,56,57). It is only accurate, however, if the above requirements are satisfied. The performance of any reference gene considered for use as an endogenous control must be empirically determined and validated prior to experimentation. Evaluation of single reference genes is best accomplished by absolute quantitation assays, which will reveal any regulatory effects of the experimental condition on the control gene of interest.

Experimental designs and treatments that impact reference gene expression, several of which have been recently reported for classical housekeeping genes, may preclude the use of a single endogenous control gene for normalization (58,59,60). In these cases, a "basket" approach is necessary. This conservative normalization method takes the geometic mean of multiple housekeeping genes into consideration. Although it is therefore more labor intensive and costly than the single-gene approach, basket normalization minimizes the impact of treatment effects on the endogenous control set and increases the stringency of resultant data, particularly when multiple target genes are assessed (61,62). Similar to the single-gene method, stably expressed mRNAs must be empirically identified and characterized prior to inclusion in the normalization set for the basket approach to effectively minimize

noise and error (63). Basket approaches are useful when the experimental treatment causes marked changes in global gene expression or when comparing different tissue types, but may not be necessary for more straightforward experimentation.

As an alternative to endogenous reference genes as controls for normalization, PCRs can be spiked with a known amount of a synthetic RNA standard. In most cases, the synthetic standard is identical to the target gene except for a short sequence in the middle. This allows the same primer sequence to be used for both the target and the synthetic standard, which can be discriminated by size (64). Alternatively, spiking PCRs with an internal RNA standard generated with a single-point mutation ensures that the target and standard have near-identical size, sequence, and secondary structure. This approach requires post-hoc restriction digestion of the PCR products, which results in digestion of the standard and discrimination of the endogenous sequence (65). Synthetic RNA standard sequences, although extremely useful for normalization of reaction efficiencies as well as both absolute and relative quantitation, are difficult to manufacture and degrade easily, making them a more expensive and cumbersome option for normalization.

Resources

With the increasing use of real-time primer or primer/probe sets, it has become less necessary for researchers to design and test their own primer and probe sequences. Designing your own primer/probe sets for each target gene is a time-consuming process. This then has to be repeated for each organism of interest. With validated primer sets for tens of thousands of genes in many different species, researchers can either use these sequences to make their own primers and probes or they can purchase these premade from commercial suppliers (see Table 2) (66,67). This further speeds the experimental process and reduces unproductive effort optimizing primer sets. In our experience with high-throughput qPCR, we are able to obtain almost all of our primer and probe sets from prevalidated sources.

Future Technologies

With PCR in use for 25 years and qPCR approaching 20 years of age,

these techniques have been applied for a comparatively long time in biomedical research. While there are some potential replacements for qPCR, its future looks bright. The ability of RT-qPCR methods to amplify the target and thereby the increase the signal has established them as the tool of choice for quantitative analysis of the transcriptome. Future advances entail further increases in sensitivity, signal-to-noise ratios, as well as the capability to multiplex so as to analyze increasing numbers of genomic sequences in a single reaction. That qPCR is able to quantify gene expression from small sample amounts makes it an excellent technology for samples collected by new technologies like laser capture microdissection (see Reference 68). Technological development continues, primarily in making reaction volumes smaller and increasing throughput. High-throughput adaptations of real-time qPCR using nanoliter reaction volumes have been introduced (69,70). Approaches such as these offer the promise of continued use of qPCR for gene expression analysis for a number of years.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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