Review

Quantitative RT-PCR: Pitfalls and Potential

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ABSTRACT

Reverse transcription PCR (RT-PCR) represents a sensitive and powerful tool for analyzing RNA. While it has tremendous potential for quantitative applications, a comprehensive knowledge of its technical aspects is required. Successful quantitative RT-PCR involves correction for experimental variations in individual RT and PCR efficiencies. This review addresses the mathematics of RT-PCR, choice of RNA standards (internal vs. external) and quantification strategies (competitive, noncompetitive and kinetic [real-time] amplification). Finally, the discussion turns to practical considerations in experimental design. It is hoped that this review will be appropriate for those undertaking these experiments for the first time or wishing to improve (or validate) a technique in what is frequently a confusing and contradictory field.

INTRODUCTION

Quantification of steady-state mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR) has tremendous potential; however, it is also fraught with significant pitfalls. On the positive side, the technique is exquisitely sensitive, permitting analysis of gene expression from very small amounts of RNA (even at the level of the content of a single cell). Moreover, this approach can be conducted on a large number of samples and/or many different genes in the same experiment. This enables the investigator a measure of flexibility unavailable in more traditional approaches (i.e., Northern blot, dot blot and hybridization protection assays and in situ hybridization).

Along with the power and relative ease of quantitative RT-PCR (QRT-PCR) come a number of technical hurdles to utilizing the approach in a reliable manner. Specifically, as the PCR greatly amplifies the target, errors are also amplified. As a result, variability can be very large and preclude accuracy and reliable quantification. For this reason, QRT-PCR is viewed with skepticism by many investigators and reviewers. Such concern can be obviated by thoughtful experimental design and carefully validating the technique for a given gene or a given laboratory.

Since the first reports of QRT-PCR (2,5,18,54), the use of this technique has grown at an exponential rate. Despite this growth, the ability of RT-PCR to accurately quantify mRNA levels is not universally accepted. Additionally, as is typical of most emerging research areas, the terminology and theoretical basis for this technique have become muddled. This review will attempt to clarify the terminology and to describe those elements needed for accurate, precise and reproducible quantification. The basics of RT-PCR will be described first, followed by the mathematical underpinnings of RT-PCR. A subsequent discussion of RNA standard construction and methods of use will lead to considerations of the relative merits of competitive vs. noncompetitive QRT-PCR and quantification by real-time monitoring of reaction kinetics. Finally, concerns of RNA isolation, reaction phase, heteroduplex formation and absolute quantification will be reviewed.

This presentation is intended to provide the background and theoretical basis for the disparate approaches to QRT-PCR. The overview will assist an investigator in the choice of the best research tools and methods for validating the technique. A number of good reviews and books exist (8,13–15,64,66) that address many of the concerns in quantitative PCR. However, recent developments in QRT-PCR and the special issues of quantifying mRNA (as opposed to DNA) warrant a fresh look at the technique.

RT-PCR BASICS

Gene quantification has been hampered by the lack of fast, reliable and accurate methods. Northern blotting (1) works well for quantification, but it can require a large amount of total RNA and is time-consuming. The advent of PCR in 1986 (31) and the combination of reverse transcription and PCR (44,45) quickly led to the use of RT-PCR for mRNA quantification (2,18,54). Ten

years since its advent however, QRT-PCR technology is still being optimized.

Reverse Transcription

Figure 1 presents the steps in a QRT-PCR approach. Isolation of RNA (either total RNA or polyadenylated RNA) from samples can be accomplished using a number of methods [most commonly with a guanadinium-based chaotropic agent (6)]. Specific concerns relating to RNA isolation will be discussed in the RNA Isolation section.

The initial step in RT-PCR is the production of a single-strand complementary DNA copy (cDNA) of the RNA through the action of the retroviral enzyme, reverse transcriptase. Two main types of enzyme are commercially available: Moloney murine leukemia virus (MMLV-RT) and avian myeoblastosis virus (AMV-RT). The choice is largely a matter of personal preference or cost, but some direct comparisons have been reported (4). An additional, thermostable reverse transcriptase/DNA polymerase exists (r*Tth*), but it is not widely used and there is insufficient data in the literature to give a complete comparison.

An oligonucleotide primer is required to initiate cDNA synthesis. The primer anneals to the RNA, and the cDNA is extended toward the 5′ end of the mRNA through the RNA-dependent DNA polymerase activity of reverse transcriptase. Primers can be either gene-specific or nonspecific; both have advantages and disadvantages. Random hexamer primers contain all possible nucleotide combinations of a 6-base oligonucleotide and bind to all RNAs present. Similarly, oligonucleotides consisting solely of deoxythymidine residues [oligo(dT)] anneal to the polyadenylated 3′ tail found on most mRNAs. RT reactions primed by random hexamers and oligo(dT) primers can be split into a number of different PCRs, each with different gene-specific primers. This method maximizes the number of genes that can be assayed from a small RNA sample.

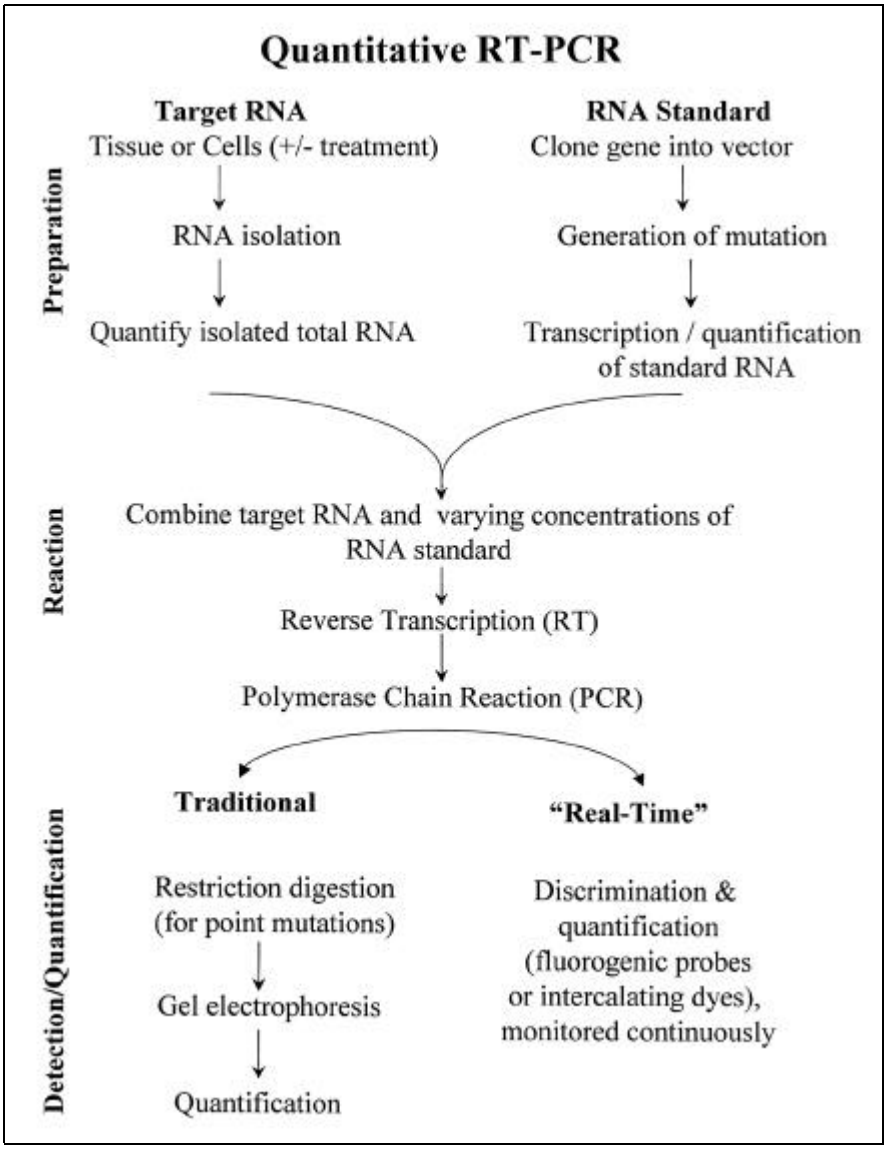

Figure 1. Flowchart of the technical steps in QRT-PCR.

Alternatively, a gene-specific primer can be used for the RT reaction. For some genes, especially rare messages, the use of sequence-specific primers increases specificity and decreases background associated with other types of primers. These gene-specific RT primers work well in conjunction with elevated RT reaction temperatures to eliminate spurious transcripts (16). These (antisense) primers can then be used for the subsequent PCR in conjunction with the corresponding gene-specific forward (sense) primer (40,53). More detailed descriptions of PCR primer design have been published (10,49). Note that with gene-specific primers, a separate RT reaction must be carried out for each gene of interest, which is unlike nonspecific primed protocols.

The RT step is the source of most of the variability in a quantitative RT-PCR experiment. The reverse transcriptase enzyme is sensitive to salts, alcohols or phenol remaining from the RNA isolation. Compounding this problem, a biphasic relationship between salt and RT-PCR amplification has been observed by this laboratory. Under some conditions, low concentrations of added salt (≤10 mM) enhance signal, and high levels of salt (≥50 mM) decrease the output signal (W.M. Freeman and K.E. Vrana, unpublished results). Therefore, salt contamination, carried over from the RNA precipitation step, can affect the apparent RNA levels. Inter-tube and inter-experiment variability are therefore common for RT reactions. This signal output vari-

ability is a central issue in QRT-PCR. It cannot be assumed that different reactions have the same RT efficiency. If one can minimize the nonspecificity and variability in this step, then the reliability of the ensuing quantification will be optimized.

PCR

Following the RT reaction, the cDNA is amplified by PCR. This discussion will not detail the specifics of the PCR; refer to other references (11,32). PCR is usually carried out using an aliquot of the RT reaction or by adding the necessary PCR components directly to the RT reaction (40). PCR is generally a three-step process, with denaturation, annealing and elongation steps, with temperatures that vary and are subject to a number of considerations that should be determined empirically. The number of cycles depends on the amount of target present and the efficiency of the reaction. As will be discussed in the Reaction Phase section, it is best to optimize cycle number to produce easily visualized products while remaining out of the plateau phase of the reaction.

Primer design and selection is also of primary importance in the PCR step. For instance, designing intron-spanning primers for PCR allows DNA contamination to be assessed. With this design, intron-containing DNA will give a different amplification product than will a spliced RNA. For intron-less genes, it is necessary to perform a control RT-PCR in which the reverse transcriptase is omitted. In fact, this valuable "minus-RT" control should be used in all experiments. The common problem of amplification product contamination from previous PCRs can be detected in this way.

Amplification Product Detection

The final step in QRT-PCR is the detection and quantification of amplification products. Reaction products must be separated so that target and standard can be discriminated and quantified. There are a variety of systems that can be used. The two broad classes of amplification product detection techniques are the traditional "end-point" measurements of product and "real-time" monitoring of product formation. End-point determinations analyze the reaction after it is completed, and real-time determinations monitor the reaction in the thermal cycler as it progresses. End-point product measurement can be accomplished through the use of fluorescent intercalating dyes, [i.e., ethidium bromide or SYBR® Green (46,48)] or through measurement of incorporated radioactivity by autoradiography or phosphor imaging. Hybridization-based protocols, such as Southern blots or fluorescence detection are also used. A third type of end-point product measurement uses solid-state approaches in which a bound enzyme produces fluorescence (19) or luminescence (50). Finally, several laboratories measure the production of amplification products following resolution by HPLC (20, 22,36) or capillary electrophoresis (3).

Real-time product monitoring offers the potential for improved quantification. The errors in sample manipulation for end-point quantification are minimized, and a great deal more information about the PCR is obtained from the data points for each cycle. One method takes advantage of the 5′ exonuclease activity of *Taq* DNA polymerase through the use of sequence-specific fluorogenic hydrolysis (TaqMan®) or hybridization probes (17,28,59,62). A second technique involves the use of DNA-specific dyes to visualize and discriminate DNA products with differing melting-curve profiles (46). This latter approach eliminates the need for designing and constructing fluorescent amplification product-specific probes.

MATHEMATICAL CONSIDERATIONS

An understanding of the mathematical descriptions of RT-PCR is essential to harness the full potential of the technique. QRT-PCR is inherently an indirect method of measurement. For reliable quantitative information to be extracted from this variable system, consideration of mathematical models is necessary (35,38,39,42,55). In this regard, the RT and the PCRs are entirely different types of enzymatic reactions and thus should be considered separately.

Mathematically, the reverse transcription step of RT-PCR is very basic. There is no amplification, and the sole variable is the percentage of mRNA converted into cDNA. This can be stated as in Equation 1:

$$[\text{cDNA}] = [\text{RNA}] \times \text{Efficiency} \quad \text{[Eq. 1]}$$

Efficiency is measured as the percentage of RNA transcribed into cDNA (0 = no RT; 1 = total RT of all RNA into cDNA). As stated previously, this reaction is extremely susceptible to contaminants, such that efficiency can fluctuate from 5%–90% (15). If two separate reactions have equal amounts of RNA, but their RT efficiency is unequal, the final amounts of the amplification products will be dissimilar following PCR.

The mathematical description of the PCR step of RT-PCR is more complex than that of the RT step. The amplification of cDNA by PCR is not constant like the magnification of a microscope, it differs not only for each gene or tube but also as the reaction progresses. With each cycle of temperatures, the amount of DNA is theoretically doubled. This can be expressed as in Equation 2:

$$P = T\,(1+E)^n \quad \text{[Eq. 2]}$$

where P is product (amount measured after n cycles); T is template or target (amount of cDNA from RT reaction); E is efficiency (percentage of cDNAs copied in a PCR cycle [from 0 to 1]); and n is cycle number (number of cycles through which the reaction proceeds). From Equation 2, it can be seen how small differences in amplification efficiency are compounded exponentially. Most reactions in the exponential phase have an efficiency of approximately 0.8, but this varies between reactions and for different experiments. The efficiency can also vary with the stage of reaction (discussed later). Only a 5% difference in amplification efficiency between two initially equal targets can result in one product appearing to be twice the amount of the other after 26 cycles of PCR. This example is meant to illustrate that nothing can be assumed in QRT-PCR. It would seem that RT-PCR might lack the consistency necessary for accurate quantification; however, the use of co-amplified standards can control this potential variability.

USE OF STANDARD RNA MOLECULES

The first QRT-PCR papers acknowledged the inherent variability in the technique and the need for standards (18,54). The common approach was to co-amplify a standard, either in the same tube or a separate tube. A wide range of DNA and RNA standards have been reported (for review, see References 13, 30 or 66). It is generally accepted that DNA standards are not an optimal choice because they do not compensate for the inherent variability in the RT step. Endogenous RNA standards or internal standards (actin or G3PDH) and heterologous synthetic RNAs have both been used as amplification standards. Endogenous, presumably invariant, internal standards have problems of widely differing abundance, different amplification primers and the fact that their expression is sensitive to some experimental treatments. Heterologous external standard RNAs are an improvement over endogenous standard RNAs because their levels can be controlled, but they are not homologous to the sequence of interest and are likely to have differing amplification efficiencies. Heterologous standards have been used successfully for quantification; however, we recommend that because these standards are no easier to create than a homologous standard, there is little to be gained by their use. Therefore, we will consider only the homologous, external RNA standards. A homologous synthetic RNA standard can be defined as an in vitro-transcribed synthetic RNA that shares the same primer binding sites as the native RNA and has the same intervening sequence except for a small insertion, deletion or mutation to facilitate differentiation from the native signal during quantification. Homologous RNA standards are the most suitable for two reasons: *(i)* an RNA standard (as opposed to DNA) must be used to control for variability during the RT step; and *(ii)* a homologous RNA standard is most likely to have the same or very similar RT and PCR efficiencies.

Homologous RNA standards are generally created from the entire native gene, or a portion of it, cloned into a

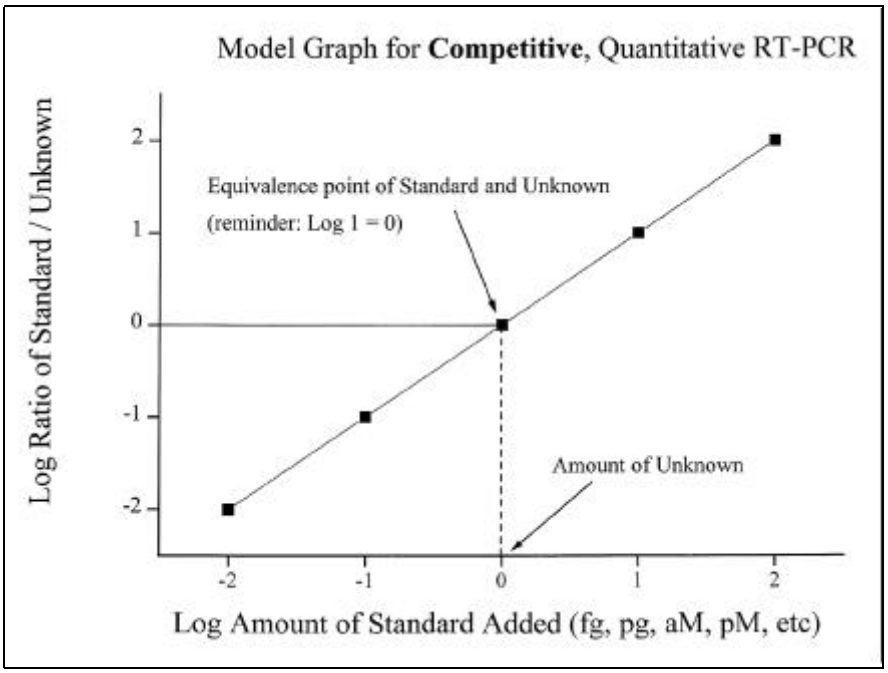

Figure 2. Theoretical analysis by competitive QRT-PCR. A constant amount of target RNA is mixed with increasing concentrations of standard RNA (in this case, over a range of 5 orders of magnitude). The mixture is subjected to RT-PCR utilizing a single set of primers. The most common representation involves plotting log (standard signal/unknown signal) vs. log standard added. When amplification product signals are equal (standard/unknown = 1), the ordinate value will be zero. This should produce a line with a slope of 1 and data points above and below the equivalence point.

plasmid containing an RNA polymerase promoter suitable for in vitro transcription. A small deletion or insertion or a mutation is made in the standard so that the native and standard amplification products can be differentiated by size on an electrophoresis gel. It has been shown that the smaller the alteration, the more similar the amplification characteristics of the standard (30). This can be explained by the fact that amplification efficiency is related to the length and secondary structure of the RNA and ensuing product. A longer product requires greater processivity of the enzyme, therefore compromising efficiency. For this reason, a balance must be found with deletion/addition style standards, where the products are easily discriminated but are as similar as possible to ensure equal efficiencies. The role/impact on RT and PCR efficiency of sequences "upstream" and "downstream" of primer binding sites has, to our knowledge, not been documented. However, it is assumed that the overall secondary structure of the RNA standard affects RT efficiency more than it will affect the PCR.

In an alternative approach, a mutation, introduced into the cloned gene by site-directed mutagenesis, can be used to create (or eliminate) a unique restriction enzyme site in the standard (2,53). After the RT-PCR, the reaction products are endonuclease-digested and subsequently resolved by electrophoresis. The signal of the two digested standard bands are summed and compared to the native signal (or vice versa if a native restriction enzyme site is eliminated in the standard).

Once the standard is constructed, the investigator must decide how best to use it. Two approaches exist for using co-amplified, external standards: competitive and noncompetitive.

Competitive RT-PCR

In competitive RT-PCR, a dilution series of the standard RNA is co-amplified with equal amounts of total RNA (and therefore equal amounts of the native gene). Competitive RT-PCR is the most common approach to quantitative RT-PCR. The standard competes with the native for primers and enzyme, thus reducing the signal of the native when the standard is in excess. As the amount of standard increases, the native signal decreases. Becker-André et al. (2) and Gilliland et al. (18) demonstrated that quantification could be achieved. From a graph of the log of standard signal/native signal vs. the log of input RNA standard, the amount of initial, native RNA can be found at the equivalence point (Figure 2). However, for competitive QRT-PCR results to be reliable, several conditions must be met. As with any standard curve, there must be standard amounts above and below the equivalence point. When the log of the standard signal/template signal is plotted against the log of the standard RNA input, a rectilinear line with a slope of 1 should be obtained (Figure 2, Reference 43). Mathematically, this slope is the ratio of the target and standard amplification efficiencies, which should be equal in all reactions. If the slope is <1, the target has a higher efficiency, whereas if the slope is >1, the standard has a higher efficiency. If the curve is not rectilinear, there is an inconsistent difference in efficiencies between the target and standard RNAs. Linearity only guarantees that there is a constant relationship between the efficiencies, and that relative levels can be determined. A slope of 1 means that the efficiencies are equal and constant, but it does not necessarily assure that absolute quantification is possible (42, 43). It has been reported that when the amount of standard in the reactions is a log dilution series, there is a decrease in the ability to detect smaller changes (51). When the amount of standard and target differ greatly, the plateau phase can be shifted and subsequent reliability is reduced (33).

Noncompetitive RT-PCR

Noncompetitive RT-PCR differs in that the native signal is unaltered by the standard. An increasing series of standard amounts is co-amplified with equal amounts of total experimental RNA; however, this occurs under conditions in which there is no competition for the components in the PCR. The quantification is therefore estimated on a linear-

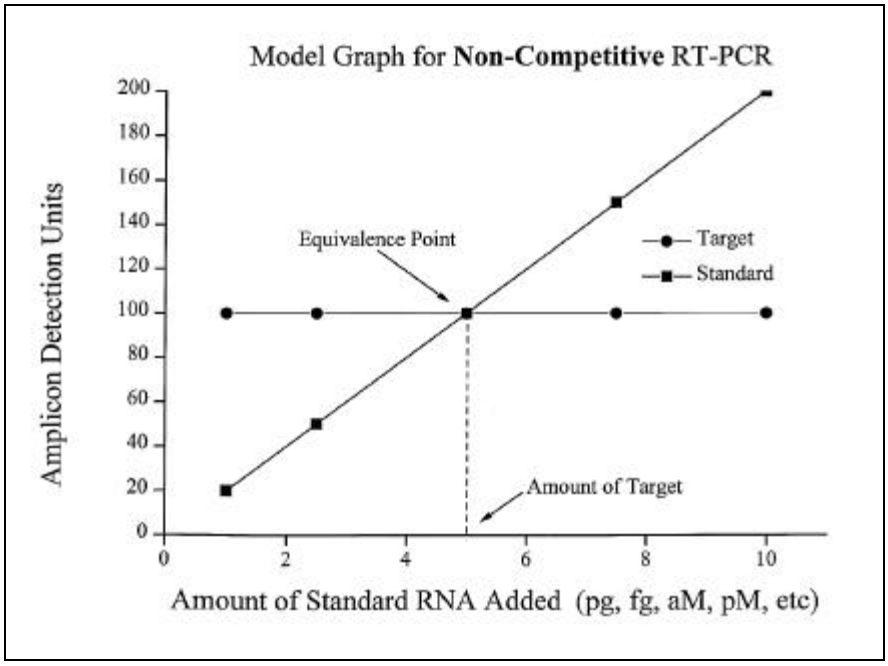

Figure 3. Theoretical analysis by noncompetitive QRT-PCR. RNAs are treated exactly as described in Figure 2 with the exception that the standard RNA is added in a linear fashion within one order of magnitude of the target. The PCRs are conducted under reagent concentrations and reaction conditions (low cycle numbers) such that no competition occurs between target and standard. The output signals of the two amplification products are plotted vs. the amount of standard RNA. To be valid, the target signal should remain constant, and the unknown curve should be linear with a slope such that a doubling of the standard input doubles the output signal. The point where standard and target lines cross is the equivalence point and the amount of the target.

scaled graph. The amount of standard signal is plotted against the native signal. When the lines intersect, they reach the equivalence point, and quantification is achieved (Figure 3). Just as in competitive RT-PCR, there must be standard amounts that are both higher and lower than the native amount. Moreover, the native signal must remain constant in all of the reactions. Generally, some estimate of the amount of native signal must be made before deciding on the standard amounts, because they are designed to differ by only one log above and below the native.

Both of these approaches have merits and disadvantages. The use of a double log scale for quantification by competitive RT-PCR reduces the quantitative reliability of the assay. As stated before, such logarithmic titration series might be unsuitable for the discrimination of very small differences in rarer messages. Relative quantification is feasible, but absolute quantification is problematic because stable differences in amplification efficiency are undetectable with competitive RT-PCR (42). Alternatively, because of the smaller range of the standard, some approximate knowledge of target amounts is essential to determine standard amounts for noncompetitive RT-PCR. The greater ability of noncompetitive RT-PCR to detect small changes may lend itself to well-characterized systems with smaller differences in native amounts, while competitive RT-PCR may be better suited for large differences in less well-characterized systems. It has been suggested that the combination of the two approaches is best where an initial competitive titration series is used to determine the approximate level of target, and then a smaller range of standards is used for quantification (34,51).

KINETIC QUANTIFICATION

The direct use of amplification kinetics to quantify RNA without the use of a standard (hence, the name kinetic RT-PCR) started as an attempt to avoid the long development times of standard construction and the problems of designing, storing and accurately quantifying the standard itself (57). While this approach has never been widely used, recent advances in detection methods suggest that this concept has the greatest potential for future quantitative RT-PCR development. This approach attempts to quantify by taking advantage of the basic mathematics of the PCR: $P = T(1 + E)^n$ (Equation 2). If treated as a simple math problem, P is derived from the amount of product as seen by any method of detection, and the number of cycles (n) is known. This leaves the reaction efficiency (E) and initial amount of target (T) to be determined. In a novel approach, Wiesner et al. (56,57) determined that by taking data points from a series of cycles, the efficiency and initial target amounts could be determined. In other words, when the amount of product from a series of cycles is plotted on a logarithmic scale against the cycle number, the slope of the line created is equal to 1 plus the reaction efficiency. The y-intercept is equal to log of the initial template concentration. In this case, the reaction efficiency is an average of the efficiencies over the cycles plotted. The specific data points for these reaction curves were obtained by removing aliquots from the reaction over a series of cycles. This technique was time-consuming and not widely used because of the need to remove and analyze aliquots from every cycle.

These logistical difficulties aside, kinetic quantification has great potential. Kinetic quantification directly computes the amplification efficiencies of different reactions. Any differences in amplification efficiency are computed and compensated for—no assumptions concerning variable PCR efficiencies are required. The amount of cDNA at the start of a reaction is computed independently for each reaction. Noticeably absent from this quantification strategy is the RT reaction efficiency, which cannot be determined in a similar manner. Initially, the question of RT reaction efficiency was sidestepped by the assumption that the RT reactions occurred with the same efficiency (56). However, as stated before, the wide differences in RT efficiency must be controlled if quantitative RT-PCR is to be accurate, precise and reproducible. A standard RNA could be added to the reactions, but the processing of multiple

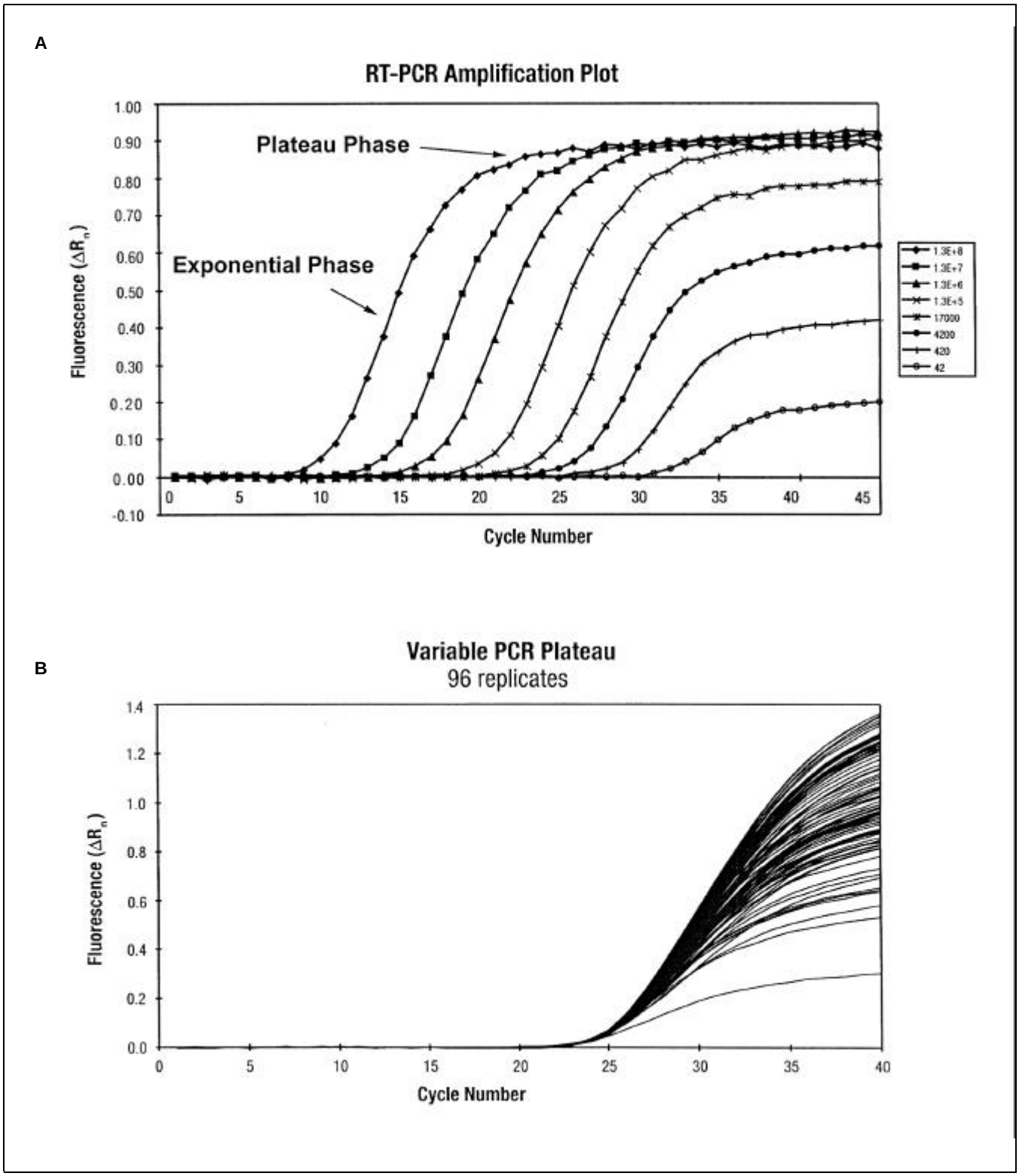

Figure 4. Exponential vs. plateau phases in PCR. (A) During the exponential phase, no factor is limiting, and the amplification products accumulate at a steady rate. At some point, reaction components become limiting, and the efficiency of amplification drops and eventually stops; this is the plateau phase. The level of the plateau phase can be independent of the initial amount of template (1.3E+5 template copies through 1.3E+8 copies gave the same plateau signal). Measurements made in the plateau are therefore not necessarily directly related to the starting material. (B) 96 replicates of the same reaction were simultaneously amplified by PCR and measured using real-time fluorescence. There is a high variability in amplification product accumulation at the plateau. Therefore, quantification at high cycle numbers is problematic. For this real-time technology, quantification at low cycle numbers is quite precise and accurate. (Modified and presented with permission from PE Applied Biosystems).

data points for each reaction is unappealing. Technological developments were therefore necessary to make this approach practical.

Over the past five years, advances in amplification product detection have made kinetic quantification feasible. If amplification products can be detected in the reaction vessels as the reaction progresses in real time, there is no need to take aliquots or conduct end-point detection schemes. Higuchi (26,27) made the first development, monitoring product accumulation by adding ethidium bromide (EtdBr) to the reactions and measuring the emitted fluorescence with a fluorometer coupled to a thermal cycler. This approach showed potential; however, EtdBr is a nonspecific double-stranded DNA (dsDNA) intercalating agent, and any measurements of fluorescence are from all sources of dsDNA and not just the signal of interest. A real-time system must be able to detect amplification product signal and to discriminate the signal of interest from background and spurious amplifications.

Two commercially available systems have been designed that both detect and discriminate amplification product signals: the ABI PRISM® 7700 and 5700 Sequence Detection Systems (PE Applied Biosystems, Foster City, CA, USA) and the LightCycler™ (Idaho Technologies, Idaho Falls, ID, USA). The PRISM 7700 System has been described in detail in a number of reports (23,59); it uses hydrolysis probes (TaqMan®; PE Applied Biosystems) to specifically detect the target sequence in the presence of nonspecific amplification products. Hydrolysis probes rely on the 5′→3′ exonuclease activity of *Taq* DNA polymerase (28,29). Fluorescent detection takes place through fiber optic lines positioned above optically non-distorting tube caps. Quantitative data are derived from a determination of the cycle at which the amplification product signal crosses a preset detection threshold. This cycle number is proportional to the amount of starting material as discussed in a number of reports (17,23, 59). Recent developments have made these instruments capable of using DNA-specific dyes and melting-curve analysis (see below).

The Light Cycler has been described fully by Wittwer et al. (60,61); it can also use fluorogenic hydrolysis or fluorogenic hybridization probes for quantification in a manner similar to the previous system (46,62). It was also, to our knowledge, the first system to quantify and differentiate products by the use of dsDNA dyes (62). This method quantifies by the re-annealing kinetics of DNA (65) and discriminates by DNA melting curves (46). Additionally, this system uses very rapid cycle times that increase throughput (52,61).

The advantage of real-time (during PCR) acquisition of data over end-point (phosphor imaging, scintillation counting, enzyme-linked immunosorbent assay [ELISA] etc.) is obvious. Fewer processing steps reduce the opportunity

for error. Moreover, the time and effort required by these manipulations are substantially reduced. The quantitative ability of this detection method comes from being able to monitor the accumulation of amplification products using either fluorogenic probes or intercalating dyes. By themselves these approaches do not control for the variability present in the RT reaction, however they logically lead toward a hybrid technique that could represent the best approach to quantitating mRNA.

Combining the power of reaction kinetics with the use of competitive or noncompetitive RNA standards could consolidate the best of both approaches and reduce the disadvantages of the individual approaches (17,62). The inclusion of synthetic RNA standards in reactions monitored by real-time technologies offers to control/compensate for most of the variability in QRT-PCR. Because recent advances allow the use of multiple fluorogenic probes in one reaction (59), the differentiation of standard and target by DNA melting curves could detect both a target and an RNA standard simultaneously. The inclusion of a unique intervening sequence in an otherwise homologous standard permits hybridization of a different fluorogenic probe for standard signal. The use of DNA melting curves could permit differentiation of single base pair mutant standards (62) and would replace the enzyme digestion required for mutated standards and associated problems. Additionally, the need for gene-specific fluorogenic probes is obviated by the use of intercalating agents like SYBR Green. A probe or signal for a reference gene, a so–called housekeeping gene, can give further validation by helping to control for differences in RNA isolation.

PRACTICAL EXPERIMENTAL CONSIDERATIONS

Additional concerns for QRT-PCR that apply to all the quantitative methods should be addressed. RNA isolation, reaction phase, heteroduplex formation and the reality of absolute quantification are all important factors in designing the best QRT-PCR protocol.

RNA Isolation and Characterization

The most common method of total RNA isolation is the guanidinium thiocyanate procedure (6). Modifications of this protocol have been developed (41), and the relative merits of this technique and others have been analyzed (63). RNA should always be subjected to denaturing agarose gel electrophoresis to visually verify the integrity of the RNA by the 28S and 18S ribosomal RNA bands. In addition, spectrophotometric measurements of RNA concentration have been reported to be sensitive to pH (58). The same denaturing gel used to confirm integrity can be used to visually verify the spectrophotometric quantification. It has also been suggested that the same RNA standards used in the RT-PCR could be added before extraction to control for differences in recovery.

Though a basic aspect of experimental design, it is worth reiterating the importance of careful sample preparation in QRT-PCR. RNA degradation is a serious problem and can lead to variable results. Though ribonuclease levels vary by organism and tissue, careful RNA isolation will enhance the quality of subsequent QRT-PCR. Additionally, if intact tissue is being used for RNA isolation, dissection must be consistent to have homogenous samples.

Reaction Phase

During the progression of a PCR, the reaction goes through two distinct phases, the exponential phase and the plateau phase (Figure 4A). In the exponential phase, theoretically, every cDNA is denatured, bound by a primer and copied by the polymerase. This phase adheres to the mathematical description previously given (Equation 2), and the amplification efficiency is consistent. This phase occurs in the early through middle cycles of a PCR and generally lasts from 10 to 20 cycles. The number of cycles before a reaction enters the exponential phase depends on the amount of starting material (compare curves in Figure 4A). Note that reporting on specific cycle numbers as comprising the exponential phase applies to only that particular reaction system and sample. Because of

differences in amount of RNA and reaction mixtures, no absolute range of cycle numbers can define the exponential phase. Rather, it must be identified experimentally for each individual system.

The exponential phase is followed by the plateau phase. In the plateau phase of the reaction, components of the reaction mixture become limiting. The supply of nucleotides might be depleted, inhibitors from the reaction might accumulate, the polymerase might lose activity or primers might become limiting. Most likely, the cDNA begins to compete for primers, and DNA amplification product concentration can increase to the point where single-stranded products re-anneal with each other rather than with a primer. The effect this has on quantification is dramatic. A reaction becomes less predictable as it enters and proceeds into the plateau phase (Figure 4B). A number of reports successfully quantify with competitive RT-PCR in the plateau phase (2,7,9,37). However, we believe this to be the exception rather than the norm. As shown in Figure 4B, equal amounts of initial template can give widely varying amounts of signal in the plateau phase but give equal signals in the early exponential phase. Differing amounts of initial template can also give equal signal in the plateau phase because they will proceed until the supply of some constant reagent is limited (Figure 4A). The main reason given for using the plateau phase is that it does not require the additional effort of showing the product accumulates linearly up to and past the cycle number in which the signal is being measured. Instead, all measurements are made at the same high cycle number. Because significant preliminary work must be conducted to establish that measurement in the plateau phase is not subject to the kind of error seen in Figure 4B, the advantage of plateau-phase quantification is negated. It is clearly prudent to conduct all quantification in the exponential phase. To ensure that quantification is being achieved in the exponential phase, a range of target and standard amounts can be amplified over a range of cycles. If the products are still accumulating in a logarithmic fashion, the system is in the exponential phase.

Heteroduplex

While a homologous standard is required to establish reaction efficiencies that are similar to the target, the similarity can also be a source of error. As the standard and target are amplified by PCR, single strands of standard and native amplification products can re-anneal to form heteroduplexes. With deletion and insertion mutants, the likelihood of heteroduplex formation is reduced (25,30). However, when a point mutant is used, heteroduplex formation is more likely. In this case, when the products are digested with enzymes, the heteroduplexes will remain undigested and indistinguishable from the standard or target, thus creating error. A double-cut method has been developed, in which both the standard and mutant contain unique restriction enzyme sites. Any heteroduplexes will remain uncut and separate from the standard and target (12,30). In addition, procedures have been developed for monitoring this potential problem and correcting for heteroduplex formation (2,21,22,25).

The need for these methods can be eliminated by minimizing heteroduplex formation. When standard amounts are in great excess of the target, the likelihood of heteroduplex formation is increased (25). Heteroduplex formation is also promoted in the plateau phase when there are not enough primers. Therefore, by performing experiments in the early exponential phase at low cycle numbers, amplification products will not have accumulated to the point where annealing kinetics favor heteroduplex formation.

Absolute Quantification Vs. Relative Quantification

To what extent is QRT-PCR quantitative? In other words, should RNA levels be stated as a difference from the control or can they be stated as an absolute quantity? Relative quantification determines the changes in steady-state expression of a gene. For the purposes of the vast majority of investigators, relative quantification is adequate. Semiquantitative is sometimes used as a synonym for relative quantification; however, it is not an optimal term because of the confusion it causes and its imprecise nature. Absolute quantification attempts to state the number of copies of a specific RNA per cell or unit mass of tissue. Absolute quantification requires a number of extra conditions and treatments that relative quantification does not. The RNA standard must be precisely quantified, but this itself poses a new series of questions (55). RNA isolation varies between samples and must somehow be controlled for. These additional questions may not be adequately resolved and certainly add more layers of difficulty.

The sensitivity of QRT-PCR in determining differences is also a matter of discussion. What percentage difference can be reliably determined? Depending on the focus of the research, either small or large differences must be distinguished. Some research areas identify 10-fold or greater changes; while in other areas, changes of 50% or more can be physiologically significant. Additionally, the sensitivity of the assay will also matter. Determining the difference between transcript levels of 10^2 and 10^3 is much more difficult than determining the difference between 10^5 and 10^6. As a QRT-PCR protocol is developed and optimized, these questions must be considered.

CONCLUSION

As detailed by this discussion, QRT-PCR is full of both problems and potentials. Great care must be taken when using QRT-PCR, but the results can be extremely valuable. As the knowledge of eukaryotic genomes expands, the opportunities to use QRT-PCR in a wide variety of settings will also expand. For instance, the changes seen with the new hybridization array technologies (24,47) will still need to be examined by QRT-PCR or northern blotting to confirm and quantify the changes identified on arrays. No other technique offers the potential to rapidly and quantitatively analyze a number of gene products from multiple small samples in a multiplex format. Clinical uses of QRT-PCR are also likely to proliferate as gene expression monitoring becomes a common diagnostic tool. QRT-

PCR is maturing and finding its niche in science, and we will continue to see a rapid growth in its use.

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