Contents

Basic principles of real-time PCR

Introduction

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Using PCR, specific sequences within a DNA or cDNA template can be copied, or "amplified", many thousand- to a million-fold. In traditional (endpoint) PCR, detection and quantitation of the amplified sequence are performed at the end of the reaction after the last PCR cycle, and involve post-PCR analysis such as gel electrophoresis and image analysis. In real-time quantitative PCR (qPCR), the amount of PCR product is measured at each cycle. This ability to monitor the reaction during its exponential phase enables users to determine the initial amount of target with great precision.

PCR theoretically amplifies DNA exponentially, doubling the number of molecules present with each amplification cycle. The number of cycles and the amount of PCR end-product can theoretically be used to calculate the initial quantity of genetic material (by comparison with a known standard), but numerous factors complicate this calculation. The ethidium bromide staining typically used to quantify endpoint PCR products prevents further amplification, and is only semiquantitative. PCR may not be exponential for the first several cycles, and the reaction eventually plateaus, so the amount of DNA should be measured while the reaction is still in the exponential amplification phase, which can be difficult to determine in endpoint PCR. To address these factors, the technique of real-time quantitative PCR was developed.

In real-time PCR, the amount of DNA is measured after each cycle by the use of fluorescent markers that are incorporated into the PCR product. The increase in fluorescent signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA (dsDNA)-binding dyes, or dye molecules attached to PCR primers or probes that are incorporated into the product during amplification.

The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction (Figure 1).

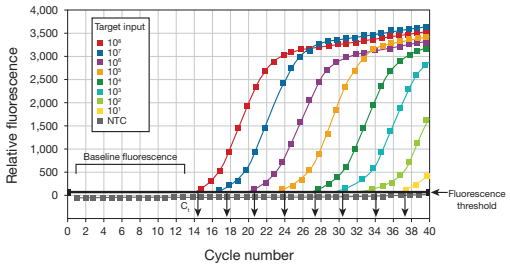


Figure 1—Amplification plots are created when the fluorescent signal from each sample is plotted against cycle number; therefore, amplification plots represent the accumulation of product over the duration of the real-time PCR experiment. The samples being amplified in this example are a dilution series of the template.

The advantages of real-time PCR include:

- → The ability to monitor the progress of the PCR reaction as it occurs in real time
- → The ability to precisely measure the amount of amplicon at each cycle
- → An increased dynamic range of detection
- → The combination of amplification and detection in a single tube, which eliminates post-PCR manipulations

Over the past several years, real-time PCR has become the leading tool for the detection and quantification of DNA or RNA. Using these techniques, you can achieve precise detection that is accurate within a two-fold range, and a dynamic range of 6 to 8 orders of magnitude.

Overview of real-time PCR

This section provides an overview of the steps involved in performing real-time PCR. Real-time PCR is a variation of the standard PCR technique used to quantify DNA or RNA in a sample. Using sequence-specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles; if the sequence is scarce, amplification is observed in later cycles. Quantification of amplified product is obtained using fluorescent probes or fluorescent DNA-binding dyes and real-time PCR instruments that measure fluorescence while performing temperature changes needed for the PCR cycles.

qPCR steps

There are three major steps that make up a qPCR reaction. Reactions are generally run for 40 cycles.

1. **Denaturation**—The temperature should be appropriate to the polymerase chosen (usually 95°C). The denaturation time can be increased if template GC content is high.
2. **Annealing**—Use appropriate temperatures based on the calculated melting temperature (T_m) of the primers (5°C below the T_m of the primer).
3. **Extension**—At 70–72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in qPCR is small, this step is often combined with the annealing step using 60°C as the temperature.

Two-step qRT-PCR

Two-step qRT-PCR starts with the reverse transcription of either total RNA or poly(A)$^+$ RNA into cDNA using a reverse transcriptase (RT). This first-strand cDNA synthesis reaction can be primed using random hexamers, oligo(dT), or gene-specific primers (GSPs). To give an equal representation of all targets in real-time PCR applications and to avoid the 3´ bias of oligo(dT), it is usually recommended that random hexamers or a mixture of oligo(dT) and random hexamers are used.

The temperature used for cDNA synthesis depends on the RT enzyme chosen. Following the first-strand synthesis reaction, the cDNA is transferred to a separate tube for the qPCR reaction. In general, only 10% of the first-strand reaction is used for each qPCR.

One-step qRT-PCR

One-step qRT-PCR combines the first-strand cDNA synthesis reaction and qPCR reaction in the same tube, simplifying reaction setup and reducing the possibility of contamination. Gene-specific primers (GSP) are required. This is because using oligo(dT) or random primers will generate nonspecific products in the one-step procedure and reduce the amount of product of interest.

Overview of qPCR and qRT-PCR components

This section provides an overview of the major reaction components and parameters involved in real-time PCR experiments. A more detailed discussion of specific components like reporter dyes, passive reference dyes, and uracil DNA glycosylase (UDG) is provided in subsequent sections of this handbook.

DNA polymerase

PCR performance is often related to the DNA polymerase, so enzyme selection is critical to success. One of the main factors affecting PCR specificity is the fact that *Taq* DNA polymerase has residual activity at low temperatures. Primers can anneal nonspecifically to DNA, allowing the polymerase to synthesize nonspecific product. The problem of nonspecific products resulting from mispriming can be minimized by using a "hot-start" enzyme. Using a hot-start enzyme ensures that no active *Taq* is present during reaction setup and the initial DNA denaturation step.

Reverse transcriptase

The reverse transcriptase (RT) is as critical to the success of qRT-PCR as the DNA polymerase. It is important to choose an RT that not only provides high yields of full-length cDNA but also has good activity at high temperatures. High-temperature performance is also very important for tackling RNA with secondary structure or when working with gene-specific primers (GSPs). In one-step qRT-PCR, an RT that retains its activity at higher temperatures allows you to use a GSP with a high melting temperature (T_m), increasing specificity and reducing background.

dNTPs

It is recommended that both the dNTPs and the *Taq* DNA polymerase be purchased from the same vendor, as it is not uncommon to see shifts of one full threshold cycle (C_t) in experiments that employ these items from separate vendors.

Magnesium concentration

In qPCR, magnesium chloride or magnesium sulfate is typically used at a final concentration of 3 mM. This concentration works well for most targets; however, the optimal magnesium concentration may vary between 3 and 6 mM.

Good experimental technique

Do not underestimate the importance of good laboratory technique. It is best to use dedicated equipment and solutions for each stage of the reactions, from preparation of the template to post-PCR analysis. The use of aerosol-barrier tips and screwcap tubes can help decrease cross-contamination problems. To obtain good replicates (ideally, triplicates), a master mix that contains all the reaction components should be prepared. The use of a master mix reduces the number of pipetting steps and, consequently, reduces the chances of cross-well contamination and other pipetting errors.

Template

Anywhere from 10 to 1,000 copies of template nucleic acid should be used for each real-time PCR reaction. This is equivalent to approximately 100 pg to 1 µg of genomic DNA, or cDNA, generated from 1 pg to 100 ng of total RNA. Excess template may increase the amount of contaminants and reduce efficiency. If the template is RNA, care should be taken to reduce the chance of genomic DNA contamination. One option is to treat the template with DNase I.

Ultrapure, intact RNA is essential for full-length, high-quality cDNA synthesis and accurate mRNA quantification. RNA should be devoid of any RNase contamination, and aseptic conditions should be maintained. To isolate total RNA, we recommend using either a column-based system such as the PureLink™ RNA Mini Kit, or TRIzol® Plus Reagent. Isolation of mRNA is typically not necessary, although incorporating this step may improve the yield of specific cDNAs. To ensure there is no genomic DNA contamination of the RNA preparation, RNA should be treated with amplification-grade DNase I prior to qRT-PCR.

Real-time PCR primer design

Good primer design is one of the most important parameters in real-time PCR. When designing gene-specific real-time PCR primers, keep in mind that the amplicon length should be approximately 80–250 bp, since longer products do not amplify as efficiently. Optimal results may require a titration of primer concentrations between 50 and 500 nM. A final concentration of 200 nM for each primer is effective for most reactions.

In general, primers should be 18–24 nucleotides in length. This provides for practical annealing temperatures. Primers should be designed according to standard PCR guidelines. They should be specific for the target sequence and be free of internal secondary structure. Primers should avoid stretches of polybase sequences (e.g., poly (dG)) or repeating motifs, as these can hybridize inappropriately to the template.

Primer pairs should have compatible melting temperatures (within 5°C) and contain approximately 50% GC content. High GC content results in the formation of stable imperfect hybrids, while high AT content depresses the T_m of perfectly matched hybrids. If possible, the 3´ end of the primer should be rich in GC bases (GC clamp) to enhance annealing of the end that will be extended. The sequences should be analyzed to avoid complementarity and prevent hybridization between primers (primer-dimers).

For qRT-PCR, design primers that anneal to exons on both sides of an intron or span an exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and potential contaminating genomic DNA by melting curve analysis. To confirm the specificity of your primers, a BLAST search may be performed against public databases to be sure that your primers only recognize the target of interest.

Primer design software

A primer design software program such as OligoPerfect™, available on the Web at www.invitrogen.com/oligoperfect, can automatically evaluate a target sequence and design primers for it based on the criteria listed above. The online version of D-LUX™ Designer software performs similar analysis when designing LUX™ primers. Many primer design software packages will automatically perform a BLAST search, comparing your sequence to similar sequences across databases to eliminate cross-reactivity.

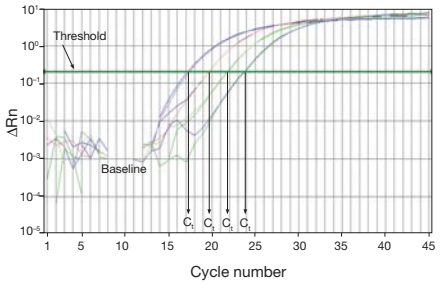


Figure 2— Log plot of amplification curves, comparing baseline, threshold, and threshold cycle (C_t) values.

At a minimum, using primer design software will ensure that primers are specific for the target sequence and free of internal secondary structure, and avoid complementary hybridization at 3´ ends within each primer and with each other. As mentioned previously, good primer design is especially critical when using DNA-binding dyes.

Real-time PCR analysis terminology

This section defines the major terms used in qPCR analysis.

Baseline

The baseline of the real-time PCR reaction refers to the signal level during the initial cycles of PCR, usually cycles 3 to 15, in which there is little change in fluorescent signal. The low-level signal of the baseline can be equated to the background or the "noise" of the reaction (Figure 2). The baseline in real-time PCR is determined empirically for each reaction, by user analysis or automated analysis of the amplification plot. The baseline should be set carefully to allow accurate determination of the threshold cycle (C_t), defined below. The baseline determination should take into account enough cycles to eliminate the background found in the early cycles of amplification, but should not include the cycles in which the amplification signal begins to rise above background. When comparing different real-time PCR experiments, the baseline should be defined in the same way for each experiment (Figure 2).

Threshold

The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish relevant amplification signal from the background. Usually, real-time PCR instrument software automatically sets the threshold at 10 times the standard deviation of the fluorescence value of the baseline. However, the positioning of the threshold can be set at any point in the exponential phase of PCR.

C_t (threshold cycle)

The threshold cycle (C_t) is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The C_t is used to calculate the initial DNA copy number, because the C_t value is inversely related to the amount of starting template. For example, in comparing two real-time PCR reactions, one with twice as much starting template as the other, the reaction with the 2X starting amount will have a C_t one cycle earlier (Figure 3). This assumes that the PCR is operating at 100% efficiency (i.e., the amount of product doubles perfectly during each cycle) in both reactions.

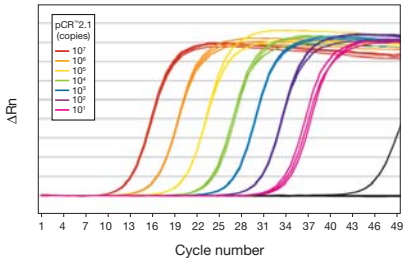


Figure 3—Amplification plots for a 10-fold dilution series. As the template amount decreases, the cycle number at which significant amplification is seen increases.

Standard curve

A dilution series of known template concentrations can be used to establish a standard curve for determining the initial starting amount of the target template or for assessing the reaction efficiency (Figure 4). The log of each known concentration in the dilution series (x-axis) is plotted against the C_t value for that concentration (y-axis). From this standard curve, information about the performance of the reaction as well as various reaction parameters (including slope, y-intercept, and correlation coefficient) can be derived. The concentrations chosen for the standard curve should encompass the expected concentration range of the target.

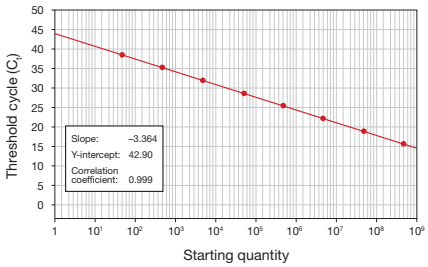


Figure 4—Example of a standard curve. A standard curve shows threshold cycle (C_t) on the y-axis and the starting quantity of RNA or DNA on the x-axis. Slope, y-intercept, and correlation coefficient values are used to provide information about the performance of the reaction.

Correlation coefficient (R^2)

The correlation coefficient is a measure of how well the data fit the standard curve. The R^2 value reflects the linearity of the standard curve. Ideally, $R^2 = 1$, although 0.999 is generally the maximum value.

Y-intercept

The y-intercept corresponds to the theoretical limit of detection of the reaction, or the C_t value expected if the lowest copy number of target molecules denoted on the x-axis gave rise to statistically significant amplification. Though PCR is theoretically capable of detecting a single copy of a target, a copy number of 10 is commonly specified as the lowest target level that can be reliably quantified in real-time PCR applications. This limits the usefulness of the y-intercept value as a direct measure of sensitivity. However, the y-intercept value may be useful for comparing different amplification systems and targets.

Exponential phase

It is important to quantify your real-time PCR reaction in the early part of the exponential phase as opposed to in the later cycles or when the reaction reaches the plateau. At the beginning of the exponential phase, all reagents are still in excess, the DNA polymerase is still highly efficient, and the product, which is present in a low amount, will not compete with the primers' annealing capabilities. All of these things contribute to more accurate data.

Slope

The slope of the log–linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible, equivalent to a slope of –3.32 (see *Efficiency*, below, for more detail).

Efficiency

A PCR efficiency of 100% corresponds to a slope of –3.32, as determined by the following equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Ideally, the efficiency (E) of a PCR reaction should be 100%, meaning the template doubles after each cycle during exponential amplification. The actual efficiency can give valuable information about the reaction. Experimental factors such as the length, secondary structure, and GC content of the amplicon can influence efficiency. Other conditions that may influence efficiency are the dynamics of the reaction itself, the use of non-optimal reagent concentrations, and enzyme quality, which can result in efficiencies below 90%. The presence of PCR inhibitors in one or more of the reagents can produce efficiencies of greater than 110%. A good reaction should have an efficiency between 90% and 110%, which corresponds to a slope of between –3.58 and –3.10.

Dynamic range

This is the range over which an increase in starting material concentration gives rise to a corresponding increase in product. Ideally, this should be a 7–8 log range for plasmid DNA and at least a 3 to 4 log range for cDNA or genomic DNA.

Absolute quantification

Absolute quantification describes a qPCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. An unknown sample can then be quantified based on this curve.

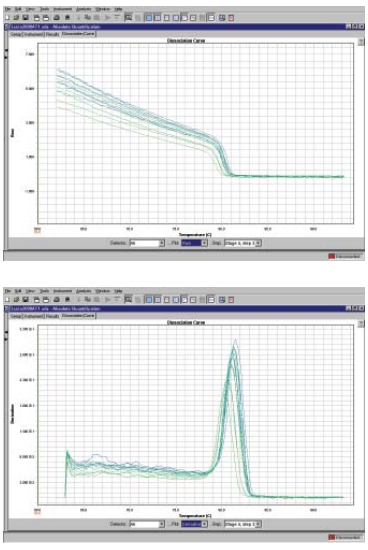


Figure 5—Melting curve (A) and –ΔF/ΔT vs. temperature (B).

Relative quantification

Relative quantification describes a real-time PCR experiment in which the gene of interest in one sample (i.e., treated) is compared to the same gene in another sample (i.e., untreated). The results are expressed as fold up- or down-regulation of the treated in relation to the untreated. A normalizer gene (such as β-actin) is used as a control for experimental variability in this type of quantification.

Melting curve (dissociation curve)

A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts", into single-stranded DNA (ssDNA) as the temperature of the reaction is raised. For example, when double-stranded DNA bound with SYBR® Green I dye is heated, a sudden decrease in fluorescence is detected when the melting point (T_m) is reached, due to dissociation of the DNA strands and subsequent release of the dye. The fluorescence is plotted against temperature (Figure 5A), and then the $-\Delta F/\Delta T$ (change in fluorescence/change in temperature) is plotted against temperature to obtain a clear view of the melting dynamics (Figure 5B).

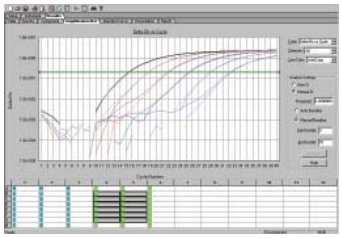
Post-amplification melting-curve analysis is a simple, straightforward way to check real-time PCR reactions for primer-dimer artifacts and contamination and to ensure reaction specificity. Because the melting temperature

of nucleic acids is affected by length, GC content, and the presence of base mismatches, among other factors, different PCR products can often be distinguished by their melting characteristics. The characterization of reaction products (e.g., primer-dimers vs. amplicons) via melting curve analysis reduces the need for time-consuming gel electrophoresis.

The typical real-time PCR data set shown in Figure 6 illustrates many of the terms that have been discussed. Figure 6A illustrates a typical amplification plot generated using LUX™ primers. During the early cycles of the PCR reaction, there is little change in the fluorescent signal. As the reaction progresses, the level of fluorescence begins to increase with each cycle. The reaction threshold is set above the baseline in the exponential portion of the plot. This threshold is used to assign the threshold cycle, or C_t value, of each amplification reaction. C_t values for a series of reactions containing a known quantity of target can be used to generate a standard curve. Quantification is performed by comparing C_t values for unknown samples against this standard curve or, in the case of relative quantification, against each other, with the standard curve serving as an efficiency check. C_t values are inversely related to the amount of starting template: the higher the amount of starting template in a reaction, the lower the C_t value for that reaction.

Figure 6B shows the standard curve generated from the C_t values in the amplification plot. The standard curve provides important information regarding the amplification efficiency, replicate consistency, and theoretical detection limit of the reaction.

A



B

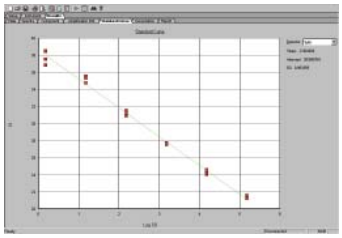


Figure 6—Amplification of β-actin from 10 to 10^7 copies. Real-time PCR of 10-fold serial dilutions of a human β-actin cDNA clone was performed using 200 nM of a FAM-labeled LUX™ primer, 200 nM of corresponding unlabeled primer, Platinum® Quantitative PCR SuperMix-UDG, and ROX Reference Dye. Reactions (3 replicates per dilution) were incubated 3 minutes at 95°C, followed by 45 cycles of 95°C, 15 sec; 60°C, 45 sec using an ABI PRISM® 7300 system. **A**. Amplification plot. **B**. Standard curve showing initial copy number of template vs. threshold cycle (C_t).

Real-time PCR fluorescence detection systems

Several different fluorescence detection technologies can be used for real-time PCR, and each has specific assay design requirements. All are based on the generation of a fluorescent signal that is proportional to the amount of PCR product formed. The three main fluorescence detection systems are:

- → DNA-binding agents (e.g., SYBR® Green and SYBR® GreenER™ technologies)
- → Fluorescent primers (e.g., LUX™ Fluorogenic Primers and Amplifluor™ qPCR primers)
- → Fluorescent probes (e.g., TaqMan® probes, Scorpions, Molecular Beacons)

The detection method plays a critical role in the success of real-time PCR.

DNA-binding dyes

The most common system for detection of amplified DNA is the use of intercalating dyes that fluoresce when bound to dsDNA. **SYBR® Green I and SYBR® GreenER™** technologies use this type of detection method.

The fluorescence of DNA-binding dyes significantly increases when bound to double-stranded DNA (dsDNA). The intensity of the fluorescent signal depends on the amount of dsDNA that is present. As dsDNA accumulates, the dye generates a signal that is proportional to the DNA concentration and can be detected using real-time PCR instruments.

This technology, while simple, can lacks specificity because the dye binds indiscriminately to all dsDNA formed during real-time PCR, not just to the target DNA. Thus, PCR artifacts such as primer-dimers and spurious amplification products may be detected and contribute to the overall fluorescent signal. Good primer design and a quality reagent system are critical to avoid formation of these nonspecific products. An advantage of many DNA-binding dyes is that they enable specificity to be assessed using melting curve analysis (see page 16).

Primer-based detection systems

Primer-based fluorescence detection technologies can provide highly sensitive and specific detection of DNA and RNA. In these systems, the fluorophore is attached to a target-specific PCR primer that increases in fluorescence when incorporated into the PCR product during amplification.

Amplifluor™ real-time PCR primers are designed with both a fluorophore and quencher on the same primer. The primer adopts a hairpin configuration that brings the fluorophore in close proximity to the quencher. The fluorescent signal increases when the primer is unfolded and the fluorophore and quencher are de-coupled during incorporation into an amplification product.

LUX™ Fluorogenic Primer Sets consist of an unlabeled primer and a primer labeled with a single fluorophore close to the 3′ end in a hairpin structure. This hairpin configuration effectively quenches the fluorescent signal prior to PCR without the need for a separate quenching moiety. When the primer is incorporated into a double-stranded PCR product, the fluorophore is dequenched, resulting in a significant increase in fluorescent signal (Figure 7). Because the fluorescence level decreases when the double-stranded DNA dissociates, LUX™ primers are compatible with melting curve analysis.

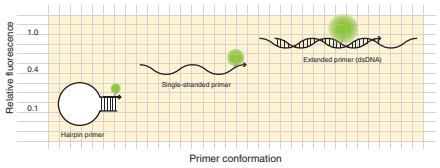


Figure 7—Conformations of LUX™ Fluorogenic Primers. The primer is labeled with a single fluorophore close to the 3′ end in a hairpin structure. When the primer is extended, the fluorophore is no longer quenched and the fluorescence signal increases.

Probe-based detection systems

Probe-based systems provide highly sensitive and specific detection of DNA and RNA. However, dual-labeling and complex design specifications make them expensive and more difficult to use than primer-based systems or DNA-binding dyes.

TaqMan® probes require a pair of PCR primers in addition to a probe with both a reporter and a quencher dye attached. The probe is designed to bind to the sequence amplified by the primers. During qPCR, the probe is cleaved by the 5′ nuclease activity of the *Taq* DNA polymerase; this releases the reporter dye and generates a fluorescent signal that increases with each cycle (Figure 8).

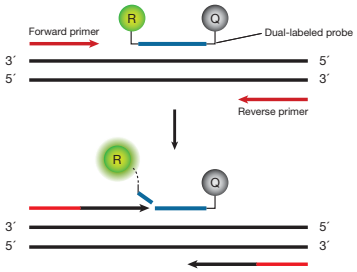


Figure 8—TaqMan® probes require a pair of PCR primers in addition to a probe with both a reporter and a quencher dye attached. When the probe is cleaved, the reporter dye is released and generates a fluorescent signal.

Melting curve analysis

Melting curve analysis and detection systems

Melting curve analysis can only be performed with real-time PCR detection technologies in which the fluorophore remains associated with the amplicon. Amplifications that have used SYBR® Green I or SYBR® GreenER™ dye or LUX™ primers can be subjected to melting curve analysis. Dual-labeled probe detection systems such as TaqMan® probes are not compatible because they produce an irreversible change in signal by cleaving and releasing the fluorophore into solution during the PCR; however, the increased specificity of this method makes this less of a concern.

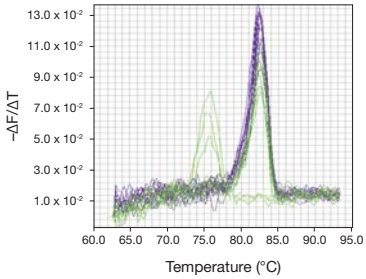


Figure 9—Melting curve analysis can detect the presence of nonspecific products, as shown by the additional peaks to the left of the peak for the amplified product in the melt curve.

The level of fluorescence of both SYBR® Green I and SYBR® GreenER™ dyes significantly increases upon binding to dsDNA. By monitoring the dsDNA as it melts, a decrease in fluorescence will be seen as soon as the DNA becomes single-stranded and the dye dissociates from the DNA.

Similarly, the fluorophore on a LUX™ primer remains linked to the PCR product, and the fluorescence decreases upon melting the dsDNA. This feature makes melting curve analysis quick and easy when LUX™ primers have been used for the qPCR reaction.

Importance of melting curve analysis

The specificity of a real-time PCR assay is determined by the primers and reaction conditions used. However, there is always the possibility that even well-designed primers may form primer-dimers or amplify a nonspecific product (Figure 9). There is also the possibility when performing qRT-PCR that the RNA sample contains genomic DNA, which may also be amplified. The specificity of the qPCR or qRT-PCR reaction can be confirmed using melting curve analysis. When melting curve analysis is not possible, additional care must be used to establish that differences observed in C_t values between reactions are valid and not due to the presence of nonspecific products.

Melting curve analysis and primer-dimers

Primer-dimers occur when two PCR primers with homologous sequences (either same-sense primers or sense and antisense primers) bind to each other instead of to the template. Melting curve analysis can identify the presence of primer-dimers because they exhibit a lower melting temperature than the amplicon. The presence of primer-dimers is not desirable in samples that contain template, as it decreases PCR efficiency and obscures analysis. The formation of primer-dimers most often occurs in no-template controls (NTCs), where the polymerase enzyme is essentially idle. Because quantitative analysis is not performed on the NTC samples, the appearance of primer-dimers is inconsequential. Melting curve analysis of NTCs can discriminate between primer-dimers and spurious amplification due to contaminating nucleic acids in the reagent components.

How to perform melting curve analysis

To perform melting curve analysis, the real-time PCR instrument can be programmed to include a melting profile immediately following the thermocycling protocol. After amplification is complete, the instrument will reheat your amplified products to give complete melting curve data (Figure 10). Most real-time PCR instrument platforms now incorporate this feature into their analysis packages.

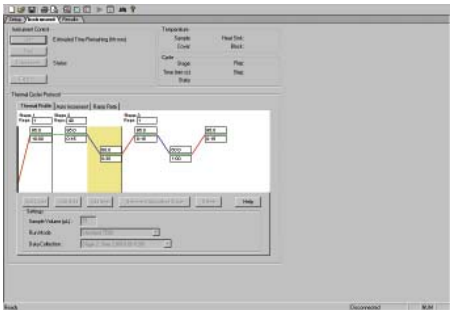


Figure 10—Example of a melting curve thermal profile setup on an Applied Biosystems instrument (rapid heating to 94°C to denature the DNA, followed by cooling to 60°C.)

In general, the program steps will be:

1. Rapid heating of the amplified sample to 94°C to denature the DNA.
2. Cooling the sample to 60°C.
3. Slowly heating (by increasing the temperature 0.2°C/second) the sample while plotting fluorescence signal vs. temperature. (As the temperature increases and the dsDNA strands melt, the fluorescence signal will decrease.)

Use of passive reference dyes

Passive reference dyes are frequently used in real-time PCR to normalize the fluorescent signal of reporter dyes and correct for fluctuations in fluorescence that are non-PCR based. Normalization is necessary to correct for fluctuations from well to well caused by changes in reaction concentration or volume, or to correct for variations in instrument scanning.

Most real-time PCR instruments use ROX as the passive reference dye, because it does not affect the real-time PCR reaction and has a fluorescent signal that can be distinguished from that of any reporter or quencher dye used. An exception is the Bio-Rad iCycler iQ® instrument system, which uses fluorescein as the reference dye.

ROX reference dye

ROX reference dye from Invitrogen is designed to normalize the fluorescent reporter signal in real-time PCR on instruments that are compatible with its use. It is an effective tool for the normalization of fluorescent reporter signal without modifying the instrument's default analysis parameters. ROX provides an internal reference to:

- → Normalize for non-PCR related fluctuations in fluorescence (e.g., caused by pipetting errors)
- → Normalize for fluctuations in fluorescence resulting from machine "noise"
- → Compensate for variations in instrument excitation and detection
- → Provide a stable baseline for multiplex qPCR and qRT-PCR

Fluorescein reference dye

Bio-Rad iCycler® instruments require the collection of "well factors" before each run to compensate for any instrument or pipetting non-uniformity. Well factors for experiments using SYBR® Green I or SYBR® GreenER™ dye are calculated using an additional fluorophore, fluorescein.

Well factors are collected using either a separate plate containing fluorescein in each well (external well factors) or the experimental plate with fluorescein spiked into the real-time PCR master mix (dynamic well factors). You must select the method when you start each run using the iCycler® instrument. The iCycler® iQ™5 and MyiQ™ systems allow you to save the data from an external well factor reading as a separate file, which can then be referenced for future readings.

Contamination prevention

As with traditional PCR, real-time PCR reactions can be affected by nucleic acid contamination, leading to false positive results. Some of the possible sources of contamination are:

- → Cross-contamination between samples
- → Contamination from laboratory equipment
- → Carryover contamination of amplification products and primers from previous PCRs; this is considered to be the major source of false positive PCR results

Uracil DNA glycosylase (UDG)

Uracil DNA glycosylase (UDG) is used to reduce or prevent DNA carryover contamination between PCR reactions by preventing the amplification of non-template DNA in real-time PCR. The use of UDG prior to a PCR reaction reduces false positives, in turn increasing the efficiency of the real-time PCR reaction and the reliability of your data.

How UDG carryover prevention works

The use of UDG for carryover prevention begins with the substitution of dUTP for dTTP in real-time PCR assays. Subsequent real-time PCR reaction mixes are then treated with UDG, which degrades any contaminating uracil-containing PCR products, leaving the natural (thymine-containing) target DNA template unaffected.

With standard UDG, a short incubation at 50°C is performed prior to the PCR cycling to allow the enzyme to cleave the uracil residues from any contaminating DNA. The removal of the uracil bases causes fragmentation of the DNA, preventing its use as a template in PCR. The UDG is then inactivated in the ramp up to 95°C in PCR. A heat-labile form of the enzyme is also available, which is inactivated at 50°C, allowing its use in one-step qRT-PCR reaction mixes.

Multiplex real-time PCR

In multiplex real-time PCR, more than one set of gene-specific primers is used to amplify separate genes from the template DNA or RNA in a single tube. Typically, multiplex reactions are used to amplify a gene of interest and a "housekeeping" gene (e.g., β-actin or GAPDH), which is used as a normalizer for the reaction. Because more than one PCR product will be quantified in the same tube, different fluorescent reporter dyes are used to label the separate primers or probes for each gene.

In comparison to standard real-time PCR, multiplex real-time PCR is more demanding and may require the optimization of several parameters. For relative expression studies (involving qRT-PCR), the primer concentration for the internal control gene may be lower than that for the sample gene, to compensate for the difference in abundance of the two genes.

To minimize the optimization required for multiplexing experiments, select similar amplicon sizes for both the gene of interest and the reference gene. This avoids competition in favor of a smaller PCR product. Additionally, choose a reference gene with a level of expression relatively close to your gene of interest. This will ensure that the experimental conditions for both targets remain similar, even if you use limiting amounts of primers.

Additional steps to optimize the multiplex reaction can include increasing the amount of Mg^{2+} in the reaction and increasing the amount of hot-start *Taq* DNA polymerase. A primer titration is recommended for optimal results, as the optimal concentration of the reference gene primer could be between 25 nM and 500 nM. For information on data analysis for multiplex experiments, see page 51.

Internal controls and reference genes

Real-time PCR has become a method of choice for gene expression analysis. To achieve accurate and reproducible expression profiling of selected genes using real-time PCR, it is critical to use reliable internal control genes for the normalization of expression levels between experiments. The internal standard chosen as the endogenous control should be expressed at roughly the same level as the gene under evaluation. By using an endogenous control (housekeeping gene) as an active reference, quantification of an mRNA target can be normalized for differences in the amount of total RNA added to each reaction. Frequently used choices for reference genes for real-time PCR are the genes for 18S rRNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and β-actin.

Relative gene expression analysis using housekeeping genes

Relative gene expression comparisons work best when the expression level of the chosen housekeeping gene remains constant and is roughly the same as the expression level of the RNA under study. The choice of the housekeeping reference gene has recently been reviewed (Suzuki et al., 2000; Bustin, 2000).

Ideally, the chosen housekeeping gene should be validated for each target cell or tissue to confirm that the expression level of the housekeeping gene remains constant at all points of the experiment. For example, GAPDH expression has been shown to be up-regulated in proliferating cells, and 18S rRNA (a ribosomal RNA species, not mRNA) may not always represent the overall cellular mRNA population. Additionally, because 18S rRNA does not have a poly(A) tail, cDNA synthesis using oligo(dT) should not be used if the 18S rRNA gene is chosen as the housekeeping gene.

Real-time PCR instrument calibration

Instrument calibration is a critical step in ensuring the proper performance of any real-time PCR platform. Calibration ensures that the integrity of the data remains consistent over time. Real-time PCR instruments should be calibrated as part of a regular maintenance regimen and prior to using new dyes for the first time. Refer to your instrument user manual for specific instructions on calibration.

Assay design

Introduction

Successful real-time PCR assay design and development are the foundation for accurate data. Up-front planning will assist in managing any experimental variability observed during the process.

Before embarking on experimental design, clearly understand the goal of the assay—specifically, what biological questions need to be answered. For example, an assay designed to determine the relative expression level of a gene in a particular disease state will be quite different from an assay designed to determine viral copy number from that same disease state. After determining the goal of your assay, it is important to identify real-time PCR experimental controls and areas for optimization.

This section describes the stages of real-time PCR assay design and implementation. We will identify sources of variability, the role they play in data accuracy, and guidelines for optimization in the following areas:

1. Target amplicon and primer design
2. Nucleic acid purification
3. Reverse transcription
4. Controls and normalization
5. Standard curve evaluation of efficiency, sensitivity, and reproducibility

Real-time PCR assay types

Gene expression profiling is a common type of real-time PCR assay in which the relative abundance of a transcript is assessed to determine gene regulation patterns between samples. In this type of assay, RNA quality, reverse transcription efficiency, real-time PCR efficiency, quantification strategy, and the choice of a normalizer gene play particularly important roles.

Viral titer determination assays can be complex to design. In general, the viral copy number in a sample is quantified using a standard curve generated using known genome equivalents or nucleic acid harvested from a titered virus control. Success with this application hinges on the accuracy of and material used for standard curve generation. Depending on the nature of the target—an RNA or DNA virus—reverse transcription and real-time PCR efficiency will play significant roles. Whether the virus is a functional virus or total virus will also influence assay design.

In **genomic profiling assays**, the genome is analyzed for duplications or deletions. The assay design, and most specifically standard curve generation, will be dictated by whether relative or absolute quantification is desired. Assay design focuses on real-time PCR efficiency and the accuracy necessary to discriminate single-copy deviations.

Lastly, **allelic discrimination assays** can detect variation down to the single-nucleotide level. Unlike the methods described above, endpoint fluorescence is measured to determine the hetero- or homozygosity of a

genome. Primer and probe design play particularly important roles to ensure a low incidence of allele-specific cross-reactivity.

Amplicon and primer design considerations

Target amplicon size, GC content, location, and specificity

As will be discussed in more detail later in this guide, reaction efficiency is paramount to the accuracy of real-time PCR data. In a perfect scenario, the precise target in a PCR reaction will be completely copied with each cycle, which corresponds to 100% efficiency. Variations in efficiency are amplified logarithmically, and by the end of the reaction may result in potentially erroneous data.

One way to minimize efficiency bias is to amplify relatively short targets. Amplifying a 100 bp region is more likely to result in complete synthesis in a given cycle than, say, amplifying a 1,200 bp target. For this reason, real-time PCR target lengths are generally in the range of 60 bp to 200 bp. In addition, shorter amplicons act as a buffer against variations in template integrity. Primers designed to amplify larger regions are less likely to anneal with the same fragment in a slightly degraded nucleic acid sample.

Amplicon GC content and secondary structure can be another cause of data inaccuracy. Less-than-perfect doubling is more likely to occur with each cycle if secondary structure lies in the path of the polymerase. Ideally, primers should be designed to anneal with, and to amplify, a region of medium (50%) GC content with no significant GC stretches. Starting with reverse transcription, it is best to locate the amplicon near the 3′ end the transcript. If RNA secondary structure prohibits full-length cDNA synthesis in a percentage of the transcripts, these amplicons are less likely to be impacted (Figure 11).

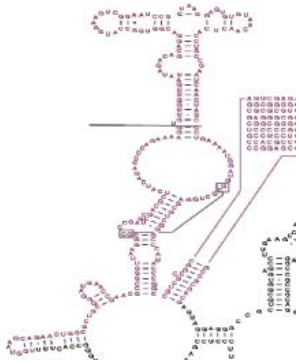


Figure 11—An RNA molecule with a high degree of secondary structure.

Amplicon specificity is another important factor in data accuracy. When designing real-time PCR primers, steps should be taken to ensure that similar sequences elsewhere in the samples are not co-amplified. Primer design software programs automate the process of screening target sequences against the originating genome and masking homologous areas, thus eliminating primer designs in these locations.

Genomic DNA, pseudogenes, and allele variants

Genomic DNA carryover in an RNA sample is a concern when profiling expression levels. The gDNA may be co-amplified with the target transcripts of interest, resulting in invalid data. Genomic DNA contamination is detected by setting up control reactions that do not contain reverse transcriptase; if the C_t for the control is higher than the C_t generated by the most dilute target, this means that the gDNA is not contributing to signal generation. However, the efficiency of the reaction may still be compromised due to competition for reaction components such as dNTPs and primers.

The best method for avoiding gDNA interference in real-time PCR is thoughtful primer design, which takes into account the introns present in gDNA that are absent in mRNA. If both real-time PCR primers are designed to anneal within the same exon, they will amplify the same region in both DNA and RNA. However, if primers are designed to anneal in separate exons, the extension reaction would have to pass through an intervening intron—which can be thousands of base pairs long—to generate an amplicon from the gDNA. Similarly, one of the primers can be designed to span an exon/exon junction, so that the 5′ end of the primer anneals in one exon and the 3′ end of the same primer anneals in the adjacent exon. This reduces the likelihood that the primer will anneal to the gDNA, though there is a chance that the 3′ end of the primer will anneal to the DNA anyway, thus allowing a completed amplicon.

Pseudogenes, or silent genes, are other transcript variants to consider when designing primers. These are derivatives of existing genes that have become nonfunctional due to mutations and/or rearrangements in the promoter or gene itself. Primer design software programs can perform BLAST searches to avoid pseudogenes and their mRNA products.

Allele variants are two or more unique forms of a gene that occupy the same chromosomal locus. Transcripts originating from these variants can vary by one or more mutations. Allele variants should be considered when designing primers, depending on whether one or more variants are being studied. In addition, GC content differences between variants may alter amplification efficiencies and generate separate peaks on a melt curve, which can be incorrectly diagnosed as off-target amplification. Alternately spliced variants should also be considered when designing primers.

Specificity, dimerization, and self-folding in primers and probes

Primer-dimers are most often caused by an interaction between forward and reverse primers, but can also be the result of forward–forward or reverse–reverse primer annealing, or a single primer folding upon itself. Primer-dimers are of greater concern in more complex reactions such as multiplex real-time PCR. If the dimerization occurs in a staggered manner, as often is the case, some extension can occur, resulting in products that approach the size of the amplicon and that become more prevalent as the cycling progresses. Typically, the lower the amount of starting template in a PCR reaction, the more likely primer-dimer formation will be. The positive side of this potential problem is that primer-dimers are usually a less favorable interaction than the desired primer–template interaction, and many options exist for minimizing or eliminating this phenomenon.

The main concern with primer-dimers is that signal from the dimers will be blended with the signal generated from the unknown targets, thereby skewing the results. This is of particular concern with reactions that use DNA-binding dyes such as SYBR® Green I dye. Another problem is that the resulting competition between primer-dimer formation and amplicon generation can contribute to a reaction efficiency outside the desirable range of 90–110%. The last major concern, also related to efficiency, is that the dynamic range of the reaction may shrink, impacting reaction sensitivity. Even if signal is not generated from the primer-dimers themselves (as is the case with fluorogenic probes), efficiency and dynamic range may still be affected.

Several free software programs are available to analyze your qPCR primer designs and determine if they will be prone to dimerize or fold upon themselves. The AutoDimer program (authored by P.M. Vallone, National Institute of Standards and Technology, USA) is a bioinformatics tool that can analyze a full list of primers at the same time (Figure 12). This is especially helpful with multiplexing applications. However, while bioinformatics analysis of primer sequences can greatly minimize the risk of dimer formation, it is still necessary to monitor dimerization experimentally.

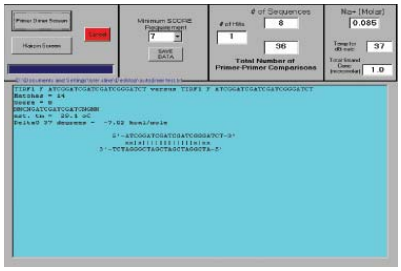


Figure 12—A screen capture from AutoDimer software. This software is used to analyze primer sequences and report areas of potential secondary structure within primers (which could lead to individual primers folding on themselves) or stretches of sequence that would allow primers to anneal to each other.

The traditional method of screening for dimers is gel electrophoresis, in which dimers appear as diffuse, smudgy bands near the bottom of the gel (Figure 13). One concern with gel validation is that it is not very sensitive and therefore may be inconclusive. However, gel analysis is useful for validating data obtained from a melting/dissociation curve, which is the most desirable method for determining the presence of dimers.

Melting curves should be generated following any real-time PCR run that uses DNA-binding dyes. In this thermal stage, the instrument ramps from low temperature, in which fluorescence is high, to high temperature, in which fluorescence is low. A sharp decrease in fluorescence will be observed at the T_m for each product generated during the PCR. The melting curve peak obtained for your no-template control (NTC) can be compared to the peak obtained from the target to determine whether dimers are present in the reaction.

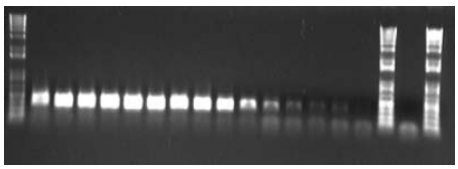


Figure 13—Agarose gel analysis to investigate primer-dimer formation. Prior to the thermal cycling reaction, the nucleic acid sample was serially diluted and added to the components of a PCR mix, and the same volume from each mixture was loaded on an agarose gel. Dimers appear as diffuse bands at the bottom of the gel.

Ideally, a single distinct peak should be observed for each reaction containing template, and no peaks should be present for the NTCs. Smaller, broader peaks at a lower melting temperature than that of the desired amplicon and also appearing in the NTC reactions are quite often dimers. Again, gel runs of product can often validate the size of the product corresponding to the melting peak.

There are situations in which dimers are present but may not affect the overall accuracy of the real-time PCR assay. A common observation is that primer-dimers are present in the no-template control but then disappear upon template addition. This is not surprising because in the absence of template, primers are much more likely to interact with each other. When template is present, dimer formation is disfavored. As long as the peak in the NTC is absent in the plus-template dissociation curve, dimers are not an issue.

Primer-dimers are part of a broad category of nonspecific products that includes amplicons created when a primer anneals to an unexpected location with an imperfect match. Amplification of nonspecific products is of concern because they can contribute to fluorescence, which in turn artificially shifts the C_t of the reaction. They can influence reaction efficiency through competition for reaction components, resulting in a decreased dynamic range and decreased data accuracy. Nonspecific products are an even greater

concern with absolute quantification assays in which precise copy numbers are reported.

Standard gel electrophoresis is generally the first step in any analysis of real-time PCR specificity. While it can help to identify products that differ in size from your target amplicon, a band may still mask similar-sized amplicons and has limited sensitivity. Due to its accuracy and sensitivity, melt curve analysis provides the most confidence in confirming gel electrophoretic assessment of primer specificity.

While nonspecific amplification should always be eliminated, if possible, there are some cases in which the presence of these secondary products is not always a major concern. For example, if alternate isoforms or multiple alleles that differ in GC content are knowingly targeted, multiple products are expected.

Primer design considerations

The following recommendations are offered for designing primers for qPCR. Note that a good primer design software program, such as the web-based OligoPerfect™ Designer (Invitrogen), can automatically design primers for specific genes or target sequences using algorithms that incorporate these guidelines, and will also perform genome-wide BLAST searches for known sequence homologies.

- → In general, design primers that are in the range of 18 to 28 nucleotides in length.
- → Avoid stretches of repeated nucleotides.
- → Aim for 50% GC content, which helps to prevent mismatch stabilization.
- → Ensure that primers have compatible T_m (within 5°C of each other).
- → Avoid sequence complementarity between all primers employed in an assay and within each primer.

Nucleic acid purification and quantitation

Real-time PCR nucleic acid purification methods

Prior to performing nucleic acid purification, one must consider the source material (cells or tissue) and potential technique limitations. DNA and RNA isolation techniques vary in their ease of use, employment of organic reagents, and resulting purity with regards to carryover of DNA (in the case of RNA isolation), protein, and organic solvents.

This section will primarily discuss RNA isolation, though most of the same guidelines also hold true for DNA isolation.

Phenol-based organic extraction is a very effective method for purifying RNA from a wide variety of cell and tissue types. During sample lysis, phenol and guanidine isothiocyanate disrupt cells and dissolve cell components

while maintaining the integrity of the nucleic acids by protecting them from RNases. Chloroform is added and the mixture is separated by centrifugation, which separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase in the presence of guanidine isothiocyanate, while DNA and protein are driven into the organic phase and interphase. The RNA is then recovered from the aqueous phase by precipitation with isopropyl alcohol.

This process is relatively fast and can yield high levels of RNA, but requires the use of toxic chemicals and may result in higher DNA carryover compared to other techniques. Residual guanidine, phenol, or alcohol can also dramatically reduce cDNA synthesis efficiency.

With **silica matrix column-based** methods, samples are lysed and homogenized in the presence of guanidine isothiocyanate, a chaotropic salt that protects RNA from endogenous RNases (Chirgwin et al., 1979). After homogenization, ethanol is added to the sample, and it is processed through a spin cartridge. RNA binds to the silica-based membrane in the spin cartridge, and impurities are effectively removed by washing (Vogelstein and Gillespie, 1979). The purified total RNA is eluted in water.

This method is even less time-consuming than organic extractions and does not require phenol. The total yields may not be quite as high, but the purity with regards to protein, lipids, polysaccharides, DNA, and purification reagents is generally better. Guanidine and ethanol carryover due to incomplete washing of the silica matrix can still occur and would have the same deleterious effects on cDNA synthesis efficiency.

Lastly, methods **combining organic lysis with silica columns** can offer the benefits of tissue lysis with the ease, speed, and purity of column methods. With these protocols, samples are lysed with a monophasic solution of phenol and guanidine isothiocyanate. This maintains the RNA integrity while disrupting cells and dissolving cell components. Chloroform addition followed by centrifugation separates the solution into an aqueous phase and an organic phase. The upper, aqueous phase containing RNA is transferred to another tube. Ethanol is added and the sample is then transferred to a silica column to bind the RNA. Contaminants are efficiently removed by thorough washing, and highly pure RNA is eluted in RNase-free water.

Assessing template quality

In assessing RNA quality and quantity, there are a few key points to focus on. Ensure that the A_{260}/ A_{280} ratio is between 1.8 and 2.0. A ratio below 1.8 can indicate protein contamination, which can lower reaction efficiency. The A_{260}/ A_{230} ratio is helpful in evaluating the carryover of components containing phenol rings such as the chaotropic salt guanidine isothiocyanate and phenol itself, which are inhibitory to enzymatic reactions. Assess RNA integrity on a denaturing gel or on an instrument such as the Agilent Bioanalyzer (Figure 14).

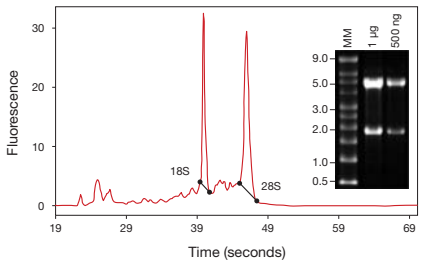


Figure 14—Agilent Bioanalyzer trace and gel image displaying RNA integrity. The two peaks in fluorescence correspond to the 18S and 28S rRNA bands.

Intact mammalian total RNA shows two bands or peaks representing the 18S and 28S rRNA species. In general, the 28S rRNA is twice as bright (or has twice the area under the peak in the Bioanalyzer trace) as the 18S rRNA. The Bioanalyzer takes RNA quality determination one step further with the assignment of a RIN (RNA integrity number) value. The RIN value is calculated from the overall trace, including degraded products, which in general is better than assessing the rRNA peaks alone. Researchers are then able to compare RIN values for RNA from different tissue types to assess quality standardization and maintenance of consistency.

Quantitation accuracy

For quantitation of RNA, fluorescent dyes such as RiboGreen® and PicoGreen® dyes are superior to UV absorbance measurements because they have higher sensitivity, higher accuracy, and high-throughput capability. UV absorbance measurements cannot distinguish between nucleic acids and free nucleotides—in fact, free nucleotides absorb more at 260 nm than do nucleic acids. Similarly, UV absorbance measurements cannot distinguish between RNA and DNA in the same sample. In addition, contaminants commonly present in samples of purified nucleic acid contribute to UV absorbance readings. Finally, most UV absorbance readers consume a considerable amount of the sample during the measurement itself. With the wide variety of fluorescent dyes available, it is possible to find reagents that overcome all of these limitations—dyes that can distinguish nucleic acids from free nucleotides, dyes that can distinguish DNA from RNA in the same sample, and dyes that are insensitive to common sample contaminants. The Qubit™ Quantitation Platform uses Quant-iT™ fluorescence technology, with advanced fluorophores that become fluorescent upon binding to DNA, RNA, or protein. This specificity means you get more accurate results than with UV absorbance readings, because Quant-iT™ Assay Kits only report the concentration of the molecule of interest (not contaminants). And, in general, quantitation methods using fluorescent dyes are very sensitive and only require small amounts of sample.

Genomic DNA carryover in expression studies

Previously we described how primer design was the first step toward eliminating DNA amplification in a real-time RT-PCR reaction. DNase treatment of the sample at the RNA isolation stage is a method by which DNA can be controlled at the source. DNase treatment can occur either in solution or on column, depending on the isolation method. On-column DNase treatments are common with silica matrix extractions, and unlike in-solution treatments they do not need to be heat-inactivated in the presence of EDTA because salt washes remove the enzyme itself. However, on-column reactions require much more enzyme.

In-solution DNase reactions must be heat-inactivated at 65°C. Free magnesium, required for the reaction, can cause magnesium-dependent RNA hydrolysis at this temperature. Therefore, EDTA is added for chelation. In most cases, the amount of EDTA used to stop the reaction is negligible when carried into a real-time PCR reaction. If there is still a concern, an equal molar amount of $MgCl_2$ may be added to the RT reaction (EDTA chelates magnesium at a 1:1 molar ratio).

Reverse transcription considerations

Reverse transcriptases

Most reverse transcriptases employed in qRT-PCR are derived from avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (M-MLV). Native AMV reverse transcriptase is generally more thermostable than M-MLV but produces lower yields. However, manipulations of these native enzymes have resulted in variants with ideal properties for qRT-PCR. An ideal reverse transcriptase will exhibit the following attributes:

→ **Thermostability**—As discussed earlier, secondary structure can have a major impact on the sensitivity of a reaction. Native RTs perform ideally between 42°C and 50°C, whereas thermostable RTs function at the higher end of (or above) this range and allow for successful reverse transcription of GC-rich regions.

→ **RNase H activity**—The RNase H domain is present in common native reverse transcriptases and functions *in vivo* to cleave the RNA strand of RNA–DNA heteroduplexes for the next stage of replication. For qRT-PCR applications, RNase H activity can drastically reduce the yield and ratio of full-length cDNA, which translates to poor sensitivity. Several RTs, most notably SuperScript® II and III, have been engineered for reduced RNase H activity.

One-step and two-step qRT-PCR

The choice between one-step and two-step qRT-PCR comes down to convenience, sensitivity, and assay design. The advantages and disadvantages of each technique must be evaluated for each experiment.

In a one-step reaction, the reverse transcriptase and *Taq* DNA polymerase are both present during reverse transcription, and the RT is inactivated in the high-temperature polymerase activation stage. Normally, the reverse transcriptase favors a buffer that is not optimal for the *Taq* DNA polymerase. Thus, the one-step buffer is a compromise solution that is not ideal for either enzyme but at the same time allows for decent functionality of both. This slightly lower functionality is compensated by the fact that all cDNA produced is amplified in the PCR stage.

The benefits of one-step qRT-PCR include the following:

- **Contamination prevention**—The closed-tube system prevents the introduction of contaminants between the RT and PCR stages.
- **Convenience**—The number of pipetting steps is reduced and hands-on time is minimized.
- **High-throughput sample screening**—For the reasons mentioned above.
- **Sensitivity**—The one-step reaction may be more sensitive than the two-step because all the first-strand cDNA created is available for real-time PCR amplification.

The drawbacks of one-step qRT-PCR are:

- **Increased risk of primer-dimers**—Forward and reverse gene-specific primers, present from the start in one-step reactions, have a greater tendency to dimerize under the 42°C to 50°C reverse transcription conditions. This can be especially problematic in reactions involving DNA-binding dyes.
- **Unavailability of cDNA for other real-time PCR reactions**—One-step reactions use all the cDNA from the RT step, so if the reaction fails, the sample is lost.

In two-step qRT-PCR, the reverse transcription is performed in an individual vial in a buffer optimized for the reverse transcriptase. Once complete, approximately 10% of the cDNA is transferred into each qPCR reaction, also in its optimal buffer.

The benefits of two-step qRT-PCR include:

- **cDNA may be archived and used for additional real-time PCR reactions**—Two-step qRT-PCR produces enough cDNA for multiple real-time PCR reactions, making it optimal for rare or limited samples.
- **Sensitivity**—The two-step reaction may be more sensitive than the one-step because the RT and qPCR reactions are performed in their individually optimized buffers.
- **Multiple targets**—Depending on the RT primers used, two-step qRT-PCR can allow you to interrogate multiple genes from a single RNA sample.

The drawbacks of two-step qRT-PCR are:

- **RT enzymes and buffers can inhibit real-time PCR**—Typically, only 10% of cDNA synthesis reaction is used in real-time PCR, because the reverse transcriptase and associated buffer components may inhibit the *Taq* DNA polymerase if not diluted properly. The specific level of inhibition will depend on the reverse transcriptase, the relative abundance of the target, and the robustness of the amplification reaction.
- **Less convenient**—Two-step reactions require more handling and are less amendable to high-throughput applications.
- **Contamination risk**—The use of separate tubes for each step increases the risk of contamination.

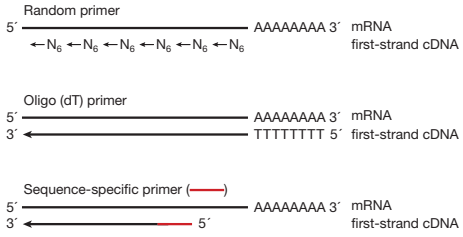


Figure 15—Graphical representation of commonly used RNA priming strategies.

RNA priming strategies

Reverse transcription has the dubious distinction of being the most variable portion of a qRT-PCR reaction. The first-strand reaction can use gene-specific primers, oligo(dT), or random primers (Figure 15), and primer selection can play a large role in RT efficiency and consistency and, consequently, data accuracy.

Random primers are great for generating large pools of cDNA, and therefore can offer the highest sensitivity in real-time PCR. They are also ideal for nonpolyadenylated RNA, such as bacterial RNA. Because they anneal throughout the target molecule, degraded transcripts and secondary structure do not pose as much of a problem as they do with gene-specific primers and oligo(dT) primers.

While increased yield is a benefit, data has shown that random primers can overestimate copy number. Employing a combination of random and oligo(dT) primers can sometimes increase data quality by combining the benefits of both in the same first-strand synthesis reaction. Random primers are used only in two-step qRT-PCR reactions.

Oligo(dT) primers are a favorite choice for two-step reactions because of their specificity for mRNA and because they allow many different targets to be studied from the same cDNA pool. However, because they always initiate reverse transcription at the 3´ end of the transcript, difficult secondary structure may lead to incomplete cDNA generation. Oligo(dT) priming of fragmented RNA, such as that isolated from FFPE samples, may also be

problematic. Nonetheless, as long as the real-time PCR primers are designed near the 3′ end of the target, premature termination downstream of this location is less of an issue.

Multiple types of oligo(dT) primers are available. Oligo(dT)$_{20}$ is a homogenous mixture of 20-mer thymidines, while oligo(dT)$_{12–18}$ is a mixture of 12-mer to 18-mer thymidines. Lastly, anchored oligo(dT) primers are designed to avoid polyA slippage by ensuring that they anneal at the 3′UTR/polyA junction. Choosing the best oligo(dT) primer may depend in part on the temperature of the reverse transcription. More thermostable RTs may perform better with longer primers, which remain more tightly annealed at elevated temperatures compared to their shorter counterparts. Oligo(dT) primers are not recommended if 18S rRNA is used for normalization.

Sequence-specific primers offer the greatest specificity and have been shown to be the most consistent of the primer options for reverse transcription. However, they do not offer the flexibility of oligo(dT) and random primers, meaning that a new cDNA synthesis reaction must be performed for each gene to be studied (unless this is a multiplex experiment, which has its own complications). This makes gene-specific primers less than optimal for processing limiting tissue or cell samples. One-step qRT-PCR reactions always employ a gene-specific primer for first-strand synthesis, while two-step reactions allow for other priming options.

While each primer type has its benefits and drawbacks, individual targets may respond differently to one primer choice over another. Therefore, each primer option should ideally be evaluated during the initial assay validation stage to determine which provides optimal sensitivity and accuracy.

Factors influencing reverse transcription efficiency

The reverse transcription stage of a qRT-PCR reaction is more inconsistent than the PCR stage. This is due to a combination of factors associated with the starting template, which the *Taq* DNA polymerase isn't normally tested with. These factors include:

- **Differences in RNA integrity**—The degradation level of a particular RNA sample has a direct impact on the percentage of mRNA target that is converted into cDNA and therefore quantified. Depending on the first-strand primer of choice, degradation may prevent the reverse transcriptase from creating cDNA across the complete stretch of RNA where the qPCR primers are designed to anneal. The lower the level of transcription efficiency, the less sensitive the assay. Efficiency variations that are not normalized can result in inaccurate conclusions.
- **GC content, RNA sample complexity, and reverse transcriptase employed**—RNA expression level comparisons are more accurate if the reverse transcriptase is less sensitive to the inevitable differences between samples. For example, data has shown that sample complexity alone—meaning all the background nucleic acid not being converted—can result in as much as a 10-fold difference in reaction

efficiency. Reverse transcriptases capable of consistently succeeding in this background are ideal.

- **Carry-over of organic solvents and chaotropic salts**—Ethanol and guanidine are necessary for RNA capture but can wreak havoc on an enzymatic reaction. Variations in the levels of these contaminants between RNA samples can affect sample comparison. Therefore, it is important to use an RNA isolation method that results in consistently low levels of these byproducts. We also recommend using a validated normalizer gene in your real-time PCR reactions.

Controls

Controls in real-time PCR reactions prove that signal obtained from the target samples are from the amplicon of interest, thereby validating specificity. All sample types benefit from a no-template control (NTC), and qRT-PCR reactions specifically require an additional no–reverse transcriptase control (no-RT).

In an NTC well, all components of a reaction are added except the template of interest. Amplification detected in these wells is due to either primer-dimers or contaminating template. Such contamination in template wells can make expression levels look artificially higher than they actually are.

In a no-RT reaction, all components except the reverse transcriptase are present. Amplification from this control indicates that DNA was present in the RNA sample. DNA can make the expression levels look artificially higher than they actually are.

Normalization methods

Earlier in this guide it was indicated that eliminating experimental inconsistencies should be the paramount concern of any real-time PCR assay design. Variations at any stage of the process will prevent the ability of researchers to compare data and will lead to erroneous conclusions if not factored out of the study. Sources of variability include the nature and amount of starting sample, the RNA isolation process, reverse transcription, and lastly real-time PCR amplification. Normalization is essentially the act of neutralizing the effects of variability from these sources. While there are individual normalization strategies at each stage of real-time PCR, some are more effective than others. These strategies include:

- **Normalizing to cell or tissue quantity**—Initiating the RNA isolation with a similar amount of tissue or cells can minimize the first variable, but this is only approximate and does not address biases in RNA isolation.
- **Normalizing to RNA quantity**—Precise quantification and quality assessment of the RNA are highly necessary, but fall short as the only

methods for normalization because they do not control for differences in efficiency in the reverse transcription and real-time PCR reactions. For example, minute differences in the levels of contaminants can affect the reverse transcription reaction, which in turn lowers the efficiency. Any variation in the RNA sample is then amplified thousands of times over, resulting in drastic fold changes unrelated to biological conditions within the cells. Pipetting is also subject to operator variation and is not normalized away with post-purification RNA analysis.

→ **Normalizing to a reference gene**—The use of a normalizer gene, (also called a reference gene or housekeeping gene) is the most thorough method of addressing almost every source of variability in real-time PCR. However, for this method to work, the gene must be present at a consistent level among all samples being compared. An effective normalizer gene controls for RNA quality and quantity, differences in reverse transcription efficiency, and real-time PCR amplification efficiency. If the reverse transcriptase transcribes or the DNA polymerase amplifies a target gene in two samples at different rates, the normalizer transcript will reflect the variability. A reference gene can be either endogenous, such as a "housekeeping" gene, or exogenous—i.e., a "spike" that is added to the sample being studied.

Endogenous normalizers

Common endogenous normalizers in real-time PCR include:

- → β-actin (BACT): cytoskeletal gene
- → 18S Ribosomal RNA (rRNA): ribosomal subunit
- → Cyclophilin A (CYC): serine-threonine phosphatase inhibitor
- → Glyceraldehyde phosphate dehydrogenase (GAPDH): glycolysis pathway
- → β-2-microglobulin (B2M): major histocompatibility complex
- → β-glucuronidase (GUS): exoglycosidase in lysomomes
- → Hypoxanthine ribosyltransferase (HPRT): purine salvage pathway
- → TATA-Box binding protein (TBP): RNA transcription

Because every real-time PCR assay is different, much thought and careful planning should go into selecting a normalizer. Instead of choosing a normalizer based on what others in the lab use, choose one that best supports the quantification strategy of the specific target gene.

The first requirement of a quality normalizer is that it is similar in abundance to your target gene. This is especially important when multiplexing, because if the normalizer plateaus before the target gene C_t is logged, the normalizer itself will cause a delayed target C_t, thus defeating its purpose.

In addition to being similar in abundance, the normalizer should have a similar amplification efficiency, as measured by the standard curve. While

correction factors can be applied when comparing reactions with different efficiencies, accuracy is enhanced when the reaction efficiencies are close to one another.

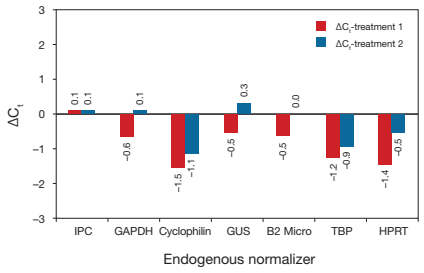


Figure 16—Gene expression levels of commonly used endogenous controls and the importance of normalization. In this example, two treatment groups and a normal group were tested for the expression levels of common reference genes, which were amplified in addition to an internal positive control (IPC). The IPC provides a standard for normal reaction-to-reaction variability. The bars represent up- or down-regulation of the normalizer in each treatment group as compared to the normal sample, which is represented by a ΔC_t of 0. The goal is to find a normalizer that mimics the changes exhibited by the IPC.

Lastly and most importantly, the normalizer should not change expression levels due to the treatment or disease state of the sample. This must be experimentally determined, as shown in the Figure 16.

While multiple replicates should be performed to ensure accuracy, it is clear that cyclophilin, TBP, and HPRT would not be good normalizer choices for these treatment groups because they are down regulated with treatment.

The expression levels of even the most common reference genes can be altered under certain conditions and therefore should always be validated:

- → GAPDH is a common normalizer and has been shown to be consistent in many cases. However, GAPDH is up regulated in some cancerous cells, in cells treated with tumor suppressors, under hypoxic conditions, and in manganese or insulin-treated samples.
- → β-actin is another commonly employed housekeeping gene because it is expressed moderately to abundantly in most cell types. However, its consistency has been questioned in breast epithelial cells, blastomeres, porcine tissues, and canine myocardium, to name a few conditions.
- → 18S ribosomal RNA constitutes 85–90% of total cellular RNA and has been shown to be quite consistent in rat liver, human skin fibroblasts, and human and mouse malignant cell lines. However, its level of abundance makes it a problematic normalizer for medium- and low-expressing targets. Often it is difficult to find a concentration of RNA at which 18S provides a wide enough baseline and also at which the target of interest generates a C_t within 40 cycles. In addition, multiplexing may

necessitate limiting the concentration of 18S primers so that the normalizer doesn't sequester all the reaction components and make conditions unfavorable for the target of interest present in the same tube. Therefore, 18S is useful only when normalizing transcripts expressed at a high level.

Alternative methods exist that don't rely on the accuracy of a single reference gene, but rather the geometric mean of multiple validated normalizers. This use of multiple consistent normalizers may prove to be a better buffer against the C_t fluctuations of any single gene, thereby increasing assay and sample-type flexibility.

Exogenous normalizers

Exogenous normalizers are not as commonly employed, but are a viable alternative if a highly consistent endogenous normalizer cannot be found for a specific sample set. An exogenous reference gene is a synthetic or *in vitro*-transcribed RNA whose sequence is not present in the samples under study. Due to its exogenous origin, it does not undergo the normal biological fluctuations that can occur in a cell under different conditions or treatments. When using this type of reference gene, the earlier it is inserted into the experimental workflow, the more steps it can control for. For example, if an exogenous transcript is added to the cell lysis buffer, it will then be present during cell lysis, RNA purification, and subsequent RT and PCR reactions.

An example of an exogenous normalizer is an *in vitro*-transcribed RNA specific to plant processes, such as a photosynthetic gene. This could be spiked into a mammalian sample because those cells would not have this same transcript endogenously.

The drawbacks to employing an exogenous normalizer are:

- → The transcript is not coming from inside the cell. The attempt is to minimize this variable by spiking the RNA into the cell lysis buffer.
- → Accuracy is subject to pipetting variability during the original introduction of the transcript.
- → Transcript stability may be affected by prolonged storage and multiple freeze-thaws. Therefore, the copy number should be routinely assessed to ensure it has not shifted over time.

Exogenous normalizers should be employed only when a viable endogenous normalizer cannot be found for a particular experiment.

Using a standard curve to assess efficiency, sensitivity, and reproducibility

The final stage before assay employment is validating that all the experimental design parameters discussed up to this point result in a highly efficient, sensitive, and reproducible experiment.

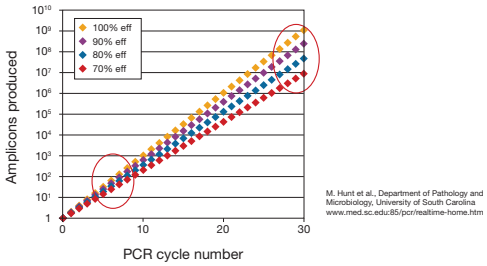


Figure 17—Bias effect caused by different amplification efficiencies. Four different qPCR reactions are shown that range from 70% to 100% efficiency. The divergence is not necessarily apparent in the early cycles. However, after 30 cycles, there is a 100-fold difference in copy number between a reaction with 70% efficiency and one with 100% efficiency. Differences in efficiency become more important in reactions with more cycles and assays that require greater sensitivity.

Reaction efficiency

As discussed previously, the overall efficiency of a real-time PCR reaction depends on the individual efficiencies of the reverse transcription reaction and the PCR amplification reaction.

Reverse transcription efficiency is determined by the percentage of target RNA that is converted into cDNA. Low conversion rates can affect sensitivity, but variations in the conversion percentage across samples is of greater concern.

PCR amplification efficiency is the most consistent factor in a real-time PCR reaction. However, this amplification exponentially magnifies slight variations in the RT stage, potentially resulting in misleading data. One hundred percent efficiency corresponds to a perfect doubling of template at every cycle, but the acceptable range is 90–110% for assay validation. This efficiency range corresponds to standard curve slopes of –3.6 to –3.1. The graph in Figure 17 shows the measurement bias resulting solely from differences in reaction efficiency.

Validating the reaction efficiency for all targets being compared (e.g., reference genes and genes of interest), optimizing those efficiencies to be as similar as possible, and employing efficiency corrections during data analysis can reduce these effects. These strategies will be discussed further in the data analysis section.

Reaction efficiency is best assessed through the generation of a standard curve. A standard curve is generated by plotting a dilution series of

template against the C_t for each dilution. The template used to generate the standard curve should match (as closely as possible) what is being used for the experiment (i.e., the same total RNA or DNA sample). The dilution range, or dynamic range, should span the concentration range expected for the unknown samples. The slope of the curve is used to determine the reaction efficiency, which most scientists agree should be between 90% and 110%.

In the following example (Figure 18), standard curves for three different targets were generated. The parallel nature of the red and blue curves indicates that they have similar efficiencies and therefore can be accurately compared at any dilution. An example of this type of comparison would be when a normalizer gene is compared against a target gene to adjust for nonbiological variability from sample to sample. The purple curve, however, becomes less efficient at the lower concentrations and therefore cannot accurately be used for comparison purposes at these lower concentrations.

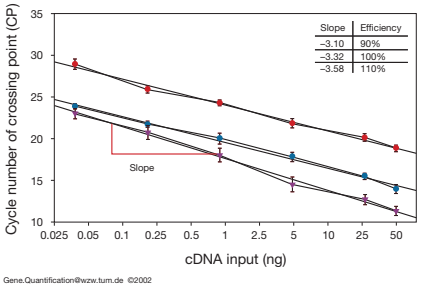


Figure 18—Illustration of amplification efficiencies between targets. Targets with similar efficiencies (parallel curves) can be accurately compared across any dilution.

In addition to assessing the experimental conditions and providing an efficiency value for relative quantity calculation, the standard curve also allows a researcher to determine whether the problem with a particular reaction is due to inhibition or a lack of optimization. This will be discussed in more detail in the *Troubleshooting* section of the handbook.

Sensitivity and reproducibility

A standard curve with an efficiency within the desirable window of 90 to 110% defines the range of input template quantities that may be measured in the real-time PCR reaction. To some, sensitivity is measured by how early a target C_t appears in the amplification plot. However, the true gauge of sensitivity of an assay is whether a given low amount of template fits to the standard curve while maintaining a desirable efficiency. The most dilute sample that fits determines reaction sensitivity.

The standard curve also includes an R^2 value, which is a measure of replicate reproducibility. Standard curves may be repeated over time to assess whether the consistency, and therefore the data accuracy for the samples, is maintained.

Data analysis

Introduction

As mentioned in the beginning of the assay design section, selecting the right quantification method depends on the goals of the experiment.

- → Absolute quantification allows actual copy numbers to be determined, but is also the most labor-intensive and difficult form of quantitation. This method requires thoughtful planning and a highly accurate standard curve. Absolute quantitation is often used for determining viral titer.
- → Comparative quantification still requires careful planning, but the data generated are for relative abundance rather than exact copy number. This is the method of choice for gene expression studies and offers two main options for quantitation: $\Delta\Delta C_t$ and standard curve quantitation.

Absolute quantification

Absolute quantification is the real-time PCR analysis of choice for researchers who need to determine the actual copy number of the target under investigation. To perform absolute quantification, a single template species of known concentration is diluted over several orders of magnitude and used to generate a standard curve in which each concentration is tied to a specific C_t value. The unknown sample C_ts are then compared to this standard curve to determine their copy number.

Standard curve generation—overview

With an absolute standard curve, the copy number of the target of interest must be known. This dictates that, prior to curve generation, the template is a pure species and is accurately quantified. Figure 19 highlights standard curve setup. A pure sample of the target of interest is accurately determined to contain 2×10^{11} copies. The sample is diluted 10-fold eight times, down to 2×10^{3} copies, and real-time PCR is performed on each dilution using at least three replicates. The resulting standard curve correlates each copy number with a particular C_t. The copy number values for the unknown samples are then derived from this standard curve. The accuracy of the quantification is therefore directly related to the quality of the standard curve.

In an absolute assay, there are a number of aspects that deserve special attention:

1. The template for standard curve generation as well as the method used to quantify that template is the foundation for the experiment. Pipetting accuracy during the dilution series is essential, but no matter how much care is taken, real-time PCR sensitivity amplifies minute human error.
2. Similar RT and PCR efficiencies for the standard template and dilution series of the actual samples are critical.

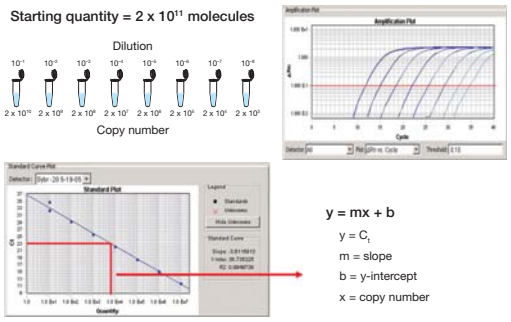


Figure 19—Workflow for standard curve setup for absolute quantification.

3. Gene-specific standard curves promote data accuracy. A new standard curve should be generated for each new gene being studied, as efficiencies will vary by target.

Standard curve generation—template choice

As mentioned, the template of choice for absolute standard curve generation will determine the accuracy of the data. While the template needs to be homogeneous and pure for initial copy number determination, the general rule is to make this template as similar to the unknown samples as possible and ensure that it is subjected to most of the same processing steps as the unknown samples. Because steps such as nucleic acid isolation and reverse transcription play a role in reaction dynamics, the efficiencies for the standards and samples will better match when this is accomplished. The following templates have been used for absolute standard curve generation:

1. **DNA standards**—PCR amplicon of the target of interest, or plasmid clone containing the target of interest
   Pros: Easy to generate, quantify, and maintain stability with proper storage.
   Cons: Avoids the reverse transcription phase of qRT-PCR, which can impact reaction efficiency significantly.
2. **RNA standards**—*In vitro*–transcribed RNA of the target of interest
   Pros: Incorporates RT efficiency and mimics the target of interest most similarly.
   Cons: Time-consuming to generate and difficult to maintain accuracy over time due to instability.

The homogeneous nature of each of the RNA and DNA standards means that they will often exhibit higher efficiencies that the actual unknown samples to be studied. Therefore, background RNA, such as yeast tRNA, can be spiked into the standard template to create a more realistic heterogeneous environment and help to balance the efficiency. It has been shown that background RNA can suppress the cDNA synthesis rate as much as 10-fold.

Standard curve application—cRNA

To demonstrate how the recommendations for absolute quantification standard curve should be applied, this section will walk step by step through the creation of a cRNA standard curve for this method of quantification.

T7 RNA polymerase can be used to generate a homogeneous pool of the transcript of interest from a plasmid or a PCR product. In this example (Figure 20), the PCR product generated from the real-time PCR itself can be reamplified with a 5´ T7 promoter–containing sequence and a 3´ poly(T)–containing reverse primer. Subsequent *in vitro* transcription produces polyadenylated sense mRNA. After purification, it is accurately quantified and diluted for the standard curve.

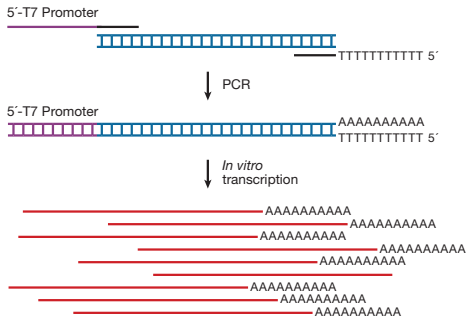


Figure 20—Schematic diagram of the *in vitro* transcription protocol. The *in vitro* transcription reaction produces polyadenylated sense mRNA. After purification, it will be accurately quantified and diluted for the standard curve.

Because it has an extended limit of detection and better accuracy, fluorometric measurement of complementary RNA (cRNA) is recommended over UV absorbance measurement (using a spectrophotometer; see Appendix for equation and explanatory notes).

With the copy number determined, unrelated yeast tRNA can be added at a 1:100 cRNA to tRNA ratio to mimic the normal background of biological samples. This standard is then diluted over at least 5 to 6 orders of magnitude for use in C_t determination by real-time PCR.

Pipetting inaccuracies can have a significant effect on absolute quantification data. Appropriate precautions can minimize this effect. As can be seen in this plate setup, three separate cRNA dilution series are prepared, and each dilution within each series is amplified in duplicate. It is important that the dilution series encompass all possible template quantities that may be encountered in the unknown samples. For example, the lowest point in the standard curve should not contain 100 template copies if it is possible that an unknown test sample may contain 10 copies.

For each dilution, six C_t values will be obtained. The high and low C_t values are discarded, and the remaining four C_t values are averaged. If we focus

on the 10^{-4} dilution in this example (Figure 21), we can see how the C_t values for a given sample vary by as many as 2 cycles. This is minimized by assigning this dilution, which corresponds to a particular copy number, the average C_t value of 21.4.

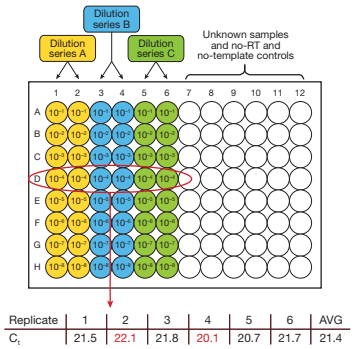


Figure 21—Plate setup for standard curve generation. When calculating the average threshold cycle (C_t) value, the highest and lowest values can be removed.

Comparative quantification

Comparative quantification, while still technically challenging, does not require the same level of stringency assigned to standard curve generation. In this technique, which applies to most gene expression studies, a gene of interest is assayed for up- or down-regulation in a calibrator (normal) sample and one or more test samples. Precise copy number determination is not necessary with this technique, which instead focuses on fold change.

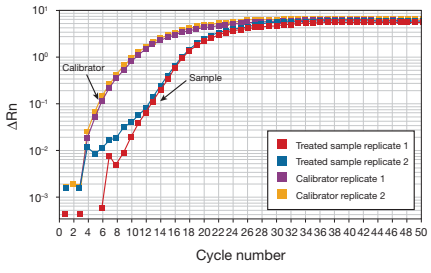
The next few pages will outline the common methods of comparative quantitation and how variability is controlled in each.

Comparative quantification algorithms—ΔC_t

This is comparative quantification in its most basic form. A C_t is obtained for the gene of interest from both a test and calibrator sample, and the difference between them is the ΔC_t. The fold difference is then simply 2 to the power of ΔC_t.

$$\text{Fold difference} = 2^{\Delta C_t}$$

The reason this basic method should not be employed is that it does not incorporate a normalizer nor standard curve for efficiency correction. If you use this method, you are making the assumptions that the same amount of input template was present in both reactions, that the sample quality was identical, and that the amplification efficiencies of the test and calibrator samples were the same.



$$\text{Fold difference} = 2^{\Delta C_t}$$

$$2^{12-6} = \text{64-fold lower in sample}$$

Figure 22—Comparative quantification of expression in a treated sample and calibrator. The treated sample and calibrator were run in duplicate; the C_t at the threshold for calibrator was 6 and the value for the treated sample was 12. The calculated relative expression level of the target gene in the treated sample is 64-fold lower than that of the calibrator.

In this example (Figure 22), the C_t of the target gene is 6 for the calibrator and 12 for the test sample. Therefore, the gene in the test sample is expressed at a level 64-fold lower than in the calibrator sample. However, how experimental variability has biased this result is unknown, mainly because a normalizer was not employed.

Comparative quantification algorithms—$\Delta\Delta C_t$

The $\Delta\Delta C_t$ method is a very popular technique that is an improvement on the ΔC_t method. With this method, C_ts for the gene of interest in both the test sample and calibrator sample are now adjusted in relation to a normalizer gene C_t from the same two samples. The resulting $\Delta\Delta C_t$ value is incorporated to determine the fold difference in expression (see ABI User Bulletin #2).

$$\text{Fold difference} = 2^{-\Delta\Delta C_t}$$

$$\Delta C_{t\ \text{sample}} - \Delta C_{t\ \text{calibrator}} = \Delta\Delta C_t$$

$$C_{t\ \text{GOI}^S} - C_{t\ \text{norm}^S} = \Delta C_{t\ \text{sample}}$$

$$C_{t\ \text{GOI}^C} - C_{t\ \text{norm}^C} = \Delta C_{t\ \text{calibrator}}$$

The requirement for the $\Delta\Delta C_t$ method is that the efficiencies for both the normalizer and target gene are identical. Of course the obvious question is: what range of deviation is acceptable? The way to determine this is to generate a standard curve for both the normalizer gene and target gene of interest using the same samples (Figure 23). The average ΔC_t between the normalizer and target gene can be obtained for each dilution. The value itself is not of concern; it is the consistency of that value across each dilution that is important.

To some researchers, this small deviation in efficiencies still opens the door to inaccuracies. Employing a correction for both the target of interest and the normalizer minimizes the effects of amplification efficiency variation.

Comparative quantification algorithms—standard curve method

The standard curve method of comparative quantification employs the C_t difference between the target gene in the test and calibrator samples, normalized to the reference gene C_ts and adjusted for minute efficiency variation. A standard curve to determine efficiency for both the normalizer and the gene of interest is necessary with this method, but it does avoid the assumptions made with previous techniques. The only requirement with this technique is that the normalizer gene be the same across all samples being compared.

Input amount RNA (ng)	Normalizer gene Avg C_t	Target gene Avg C_t	ΔC_t Normalizer - target
10	20.3	18.2	2.1
5	21.4	19.4	2
1	22.8	20.6	2.2
0.5	23.8	21.3	2.5
0.1	24.8	22.5	2.3
0.01	25.9	23.7	2.2
0.05	26.8	24.5	2.3

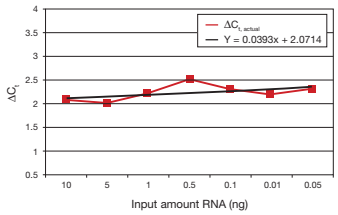


Figure 23—Relative efficiency plot. In this example, the ΔC_t ranges from 2.0 to 2.5. When plotted against dilution or input amount of RNA, a slope is obtained. While a perfectly flat line (slope = 0) indicates identical efficiency across all input concentrations, a slope of <0.1 is generally considered acceptable when employing the $\Delta\Delta C_t$ method.

$$\text{Fold difference} = (E_{target})^{\Delta C_{t\ target}} / (E_{normalizer})^{\Delta C_{t\ normalizer}}$$

E = efficiency from standard curve $\quad E = 10^{[-1\ /slope]}$

ΔC_t target = $C_{t\ GOI}{}^{c} - C_{t\ GOI}{}^{s}$

ΔC_t normalizer = $C_{t\ norm}{}^{c} - C_{t\ norm}{}^{s}$

Fold difference equation derived from M.W. Pfaffl in *A-Z of Quantitative PCR*

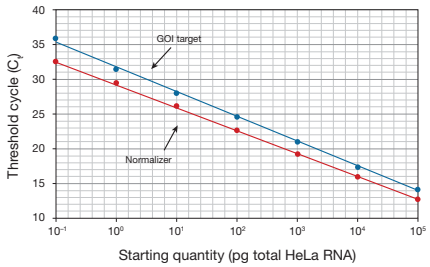


Figure 24—Efficiency values are obtained from standard curves to adjust the normalizer and gene-of-interest C_t values. HeLa RNA was diluted over 6 orders of magnitude, and real-time RT-PCR was performed to generate standard curves for both the normalizer and gene of interest (GOI).

In order to capture the most accurate efficiency value for the calculation, care should be taken in deciding what template is used as the calibrator sample for the standard curve. This template should undergo the same purification procedure, be involved in the same reactions, and have similar complexity to the unknown samples being assayed. Therefore, the perfect template is one of the heterogeneous samples containing the target of interest—for example, total RNA from the cell line or tissue being studied. Keep in mind that any differences between the calibrator sample and the unknown sample may result in an inaccurate efficiency correction and therefore inaccurate calculations for fold changes in gene expression. Because copy number is irrelevant, the dilutions or values given to those dilutions can be arbitrary.

While the same setup for the amplification curves from the $\Delta\Delta C_t$ method are used in this technique, efficiency values from standard curves (ideally run on the same plate) are now incorporated to adjust the normalizer and gene-of-interest (GOI) C_t values. Efficiencies are derived from both slopes (Figure 24).

In summary, the first step in choosing a quantification strategy is to determine whether absolute or relative quantification will best address the questions to be answered. If copy number determination is necessary, a precise standard curve using known copy numbers of template is necessary. In most cases, relative quantification will be the method of choice. The ΔC_t method does not employ a normalizer, while the $\Delta\Delta C_t$ method involves one or more reference genes to normalize for real-time PCR processing variability. With a normalizer employed, one has the option of fold-change calculations, with or without a reaction efficiency adjustment.

High-resolution melt curve (HRM) analysis

HRM is a novel homogeneous, closed-tube post-PCR method for identifying SNPs, novel mutations, and methylation patterns. HRM analysis is a more sensitive approach to traditional melt curve profiling, in which double-stranded DNA is monitored for the temperature at which it dissociates into single-stranded DNA. This temperature is known as the T_m, or melting temperature, of the product. High-resolution melt curve profiling requires a real-time PCR instrument with upgraded optical and thermal capabilities as well as analysis software for extremely fast data acquisition and highly accurate thermal control and consistency. The Rotor-Gene™ 6000 System (Corbett Life Science), 7500 System (Applied Biosystems), and LightCycler® 480 System (Roche Diagnostics) are commercially available instruments currently set up to perform HRM.

In HRM, real-time PCR is performed using a dsDNA-binding dye such as SYBR® GreenER™ dye. After 40 cycles, the real-time PCR target is amplified and highly fluorescent in an annealed state. At this stage, the HRM-capable instrument slowly ramps the temperature upward while simultaneously recording the fluorescence. The fluorescence level will slowly decrease until the temperature approaches the product T_m. Very close to the T_m, a dramatic decrease in fluorescence is observed as the sample transitions from double-stranded to single-stranded DNA (Figure 25).

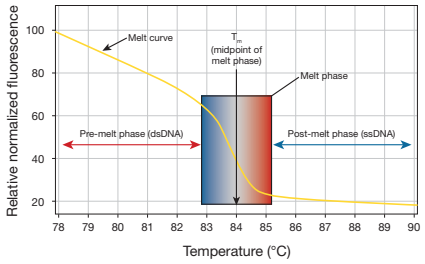


Figure 25—Characteristics of the melt curve profile for a PCR amplicon.

A specific DNA sequence has a characteristic profile. Mutations are detected as either a shift in T_m or a change in shape of the melting curve. In contrast to traditional melt curve analysis, HRM can provide single-nucleotide discrimination between amplicons. This technique has opened the door to many new applications for dsDNA-binding dyes.

HRM applications

The most widely used HRM application is gene scanning. Gene scanning is the search for the presence of unknown variations in PCR amplicons prior to, or as an alternative to, sequencing. Mutations in PCR products are detectable by HRM analysis because they lead to changes in the shape of DNA melting curves. When amplified and melted, heteroduplex DNA samples show melting curves with different profiles than those derived from homozygous wild-type or mutant samples.

Some common applications of HRM include:

- → Gene scanning (mutation discovery)
- → Mutation analysis
- → Single-nucleotide polymorphism (SNP) detection
- → Heterozygosity studies
- → Species identification
- → Methylation analysis

These applications traditionally required a unique fluorogenic probe for each target, which was expensive, time-consuming to design, and inflexible. HRM using DNA-binding dyes offers the same capabilities as probe-based analysis in a more inexpensive and flexible format.

While many applications exist for HRM, discrimination at the single-nucleotide level is one of the more challenging, due to the minute T_m shifts that must be detected.

HRM chemistry

In addition to a specialized instrument and software, HRM analysis requires dsDNA-binding dyes that are capable of distinguishing the melting points of amplicons that differ by a single nucleotide.

Dyes that have been successfully used for HRM analysis include:

- → SYTO® 9 dye (Invitrogen)
- → LCGreen® and LCGreen® Plus+ reagents (Idaho Technologies)
- → EvaGreen™ dye (Biotium Inc.)
- → SYBR® GreenER™ dye (Invitrogen)

Keys to successful HRM assays

- → Keep amplicons short for highest sensitivity. Compared to larger amplicons, those around 100 bp will allow easier detection of single-nucleotide melt events.
- → Ensure specificity of the PCR amplicon. Mispriming products and primer-dimers can complicate data interpretation. Primer concentrations lower than 200 nM, $MgCl_2$ in the 1.5 mM to 3 mM range, and use of a hot-start DNA polymerase will help to obtain high specificity. Assess mispriming using a standard (non-HRM) melt curve. No-template control (NTC) melt curves are important for evaluating specificity.
- → Avoid amplifying across regions that could contain variations other than the SNP of interest. Check for species homology, exon/intron boundaries, splice sites, and secondary structure and folding of the PCR product.
- → Maintain similar fluorescent plateaus, and therefore similar PCR product quantities, for all targets being analyzed. Differences in quantity between the samples being compared can affect melting temperatures and confound HRM analysis. Starting with similar amounts of template can be helpful.
- → Ensure that enough template is used in the reaction. In general, C_ts should be earlier than 30 to generate sufficient material for accurate melt analysis.
- → Provide a sufficient melt data collection window. For example, the window should have a range of 10°C on either side of the melting temperature of the amplicon (T_m ± 10°C). Sufficient pre- and post-melt temperature data are required for accurate curve normalization and high replicate correlation.
- → It is also recommended for some instruments to insert a pre-hold step at 50°C following amplification (but prior to melt) to ensure that all products have reassociated and to encourage heteroduplex formation. Use a mix that will provide sensitive and unbiased amplification of the genes of interest.

Data analysis for HRM

In the case of gene scanning experiment on the LightCycler® 480 System, the melting curve data will be analyzed by three different steps.

1. **Normalization**—The pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples are set to uniform, relative values from 100% to 0%. This has the effect of compensating for well-to-well temperature measurement variations.
2. **Temperature shifting**—The temperature axis of the normalized melting curves is shifted to the point where the entire double-stranded DNA is completely denatured. Samples with heterozygous SNPs can then be easily be distinguished from the wild type by the different shapes of their melting curves.

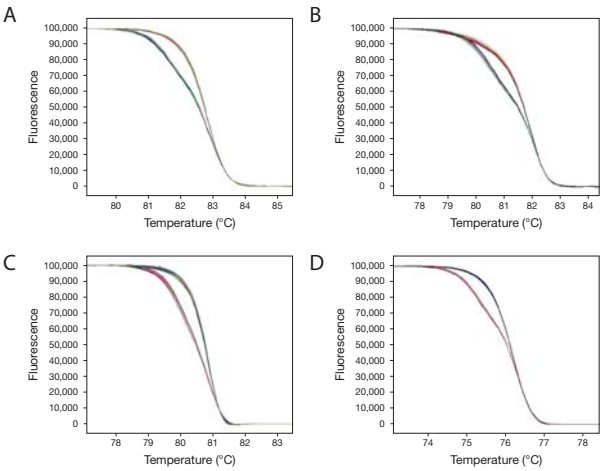


Figure 26—Fluorescence-normalized and temperature-shifted melt curves. **A.** ABCC8, SNP class 1, A/G mutation. **B.** CYP19A1, SNP class 2, C/G mutation. **C.** UGT1A1, SNP class 3, G/T mutation. **D.** ABCC1, SNP class 4, A/T mutation.

3. **Difference plots**—The differences in melting curve shape are analyzed by subtracting the curves from a reference curve. This helps cluster samples automatically into groups that have similar melting curves.

Figure 26 shows data from a gene scanning experiment on the LightCycler® 480 System. Data analysis was performed using the LightCycler® 480 Gene Scanning Software. Melt curves were fluorescence-normalized and temperature-shifted. All four assays show clear differentiation of heterozygous samples from homozygous samples.

Data analysis on the Rotor-Gene™ 6000 System proceeds in three stages:

1. **Normalization**—Allows all the curves to be compared with same starting and ending fluorescence signal level. Provides basic representation of different genotypes based on curve shifting or curve shape change.
2. **Difference plots**—Plots the difference in fluorescence between a sample and a reference at each temperature transition.
3. **Genotype calling**—Genotypes are called automatically by the software.

The same analysis sets can be used for the other HRM applications, following the recommendations of the instrument manufacturer.

Multiplex real-time PCR analysis

Multiplexing is a technique in which more than one target is analyzed in the same real-time PCR reaction. Each target is distinguished by a particular dye, conjugated to the fluorogenic probe or primer pair specific for that target. Typically, multiplex reactions are used to amplify a normalizer gene and a gene of interest in the same reaction.

The number of targets that can be amplified in a given reaction is technically limited only by the number of available spectrally distinct dyes and the number of dyes that can be excited and detected by the real-time PCR instrument. However, other experimental hurdles exist—the different primer pairs and/or probes in a reaction must not interact with each other, and these primers and probes have to share available PCR components, such as dNTPs and *Taq* DNA polymerase. While more time-consuming to optimize, multiplexing offers several distinct advantages:

- → Less variability, more consistency. Multiplexing a normalizer and gene of interest in the same tube eliminates well-to-well variability that can arise if those same two targets were amplified in different (even adjacent) wells.
- → Less reagent usage, less cost. Multiplexing requires fewer reactions to analyze the same number of targets.
- → Higher throughput. More targets can be analyzed per qPCR run and per sample.

Keys to a successful multiplex assay

A successful multiplex reaction must take many factors into consideration, including:

- → Primer and probe design
- → Reagent optimization (including primer concentration, target abundance, reaction components, and fluorophore/quencher combinations)
- → Validation of the multiplex assay

Primer and probe design

Primer and probe design is arguably the most critical factor in a multiplex assay. As reaction complexity increases, so does the probability that primers and probes will dimerize or that competition for reaction components will limit the amplification of one or more targets. The following are particularly important factors that will maximize performance while minimizing competitive effects:

- → Keep amplicons short. Designing primers to amplify a segment ranging from 60 bp to 150 bp will enhance reaction efficiency.
- → Design primers with T_ms within 5°C of each another. Remember, all primers and probes will be annealing at one temperature. Mismatched T_ms will result in an efficiency bias.

- → Perform BLAST searches with primer and probe designs to ensure their specificity for the targets of interest.
- → Use primer design software to determine whether any of the primer or probe sequences are prone to dimerize. One such free program, Auto-Dimer (authored by P.M. Vallone, National Institute of Standards and Technology, USA), can analyze all the primer designs in a given multiplex reaction for their propensity to dimerize in any combination.

Reagent optimization

A critical concern in multiplex reactions is the competition for reagents among the different targets being amplified. Ensuring a high efficiency of amplification requires either reducing the amount of primers or increasing the concentrations of all components, or both.

Primer concentration and target abundance

Not all amplicons in a multiplex reaction are present in the same numbers nor amplify at the same efficiency. Limiting the primer concentration for those targets that are easier to amplify or more abundant can level the playing field. For example, if β-actin (a high-copy normalizer) is multiplexed with a low-abundance target, it may exhaust the shared reaction components in the early cycles. Reducing the amount of β-actin primers limits its rate of amplification, allowing the less-abundant target to be amplified in an unhindered manner. In general, for higher-abundance targets, you should use the lowest primer concentration that does not delay C_ts.

Reaction component concentrations

Every multiplexing reaction is different, but increasing the amounts of *Taq* DNA polymerase, magnesium, and dNTPs and increasing the buffer strength are the changes most likely to boost the sensitivity and amplification efficiency of all targets involved.

Fluorophore/quencher combinations

The reporter fluorophores in a multiplex reaction must be spectrally distinct so that the fluorescence signal arising from each is detected in a single channel. With compatible dyes, the real-time PCR instrument excitation and emission optics are able to filter the wavelengths so that little, if any, fluorescence is attributed to the wrong fluorophore (such interference is also called "cross-talk").

Similarly, the choice of quencher for each dual-labeled fluorescent probe becomes more important as the number of probes being multiplexed increases. Fluorescent quenchers such as TAMRA, a common quencher for FAM, work by releasing the energy of the fluorophore at a different wavelength. In a multiplex reaction, quenchers of this type result in multiple signals at different wavelengths, which can complicate filtering and possibly

data integrity. Dark quenchers, on the other hand, release energy in the form of heat rather than fluorescence, and therefore keep the overall fluorescent background lower. Use the information in Table 1 as a guide for choosing compatible fluorophores and quenchers.

Choose appropriate multiplex dye combinations based on the detection capabilities of the instrument you are using. General guidelines for a few specific instruments can be found at www.biosearchtech.com/hot/multiplexing.asp. Spectral compatibility for many dyes can be found at www.invitrogen.com/spectraviewer.

Validating the multiplex assay

As with singleplex real-time PCR assays, a standard curve should be completed to assess the reaction efficiencies of all the targets in a multiplex reaction prior to running the assay. There are two main stages in this validation process:

1. Validating the primers and/or probes for each target and determining their individual efficiencies. This is the initial assessment of primer and probe design.
2. Optimizing the efficiency of the overall multiplex assay.

Evaluate each primer and probe set on an individual basis to determine the designs and conditions that are ideal for the target. This is accomplished through a dilution series standard curve, again trying to achieve close to

Table 1—Spectral information for some commonly used fluorophores and quenchers.

Dye	Excitation max (nm)	Emission max (nm)
FAM	494	518
TET	521	538
JOE	520	548
CAL Fluor® Gold 540	522	540
VIC	538	552
HEX	535	553
NED	546	575
CAL Fluor® Orange 560	538	559
Cy®3	552	570
Quasar® 570	548	566
TAMRA	560	582
CAL Fluor® Red 590	569	591
ROX	587	607
Texas Red® dye	596	615
CAL Fluor® Red 610	590	610
Cy®5	643	667
Quasar® 670	647	667

100% efficiency. After each primer/probe combination has been functionally validated, move on to multiplex optimization. It is important to keep in mind that the singleplex conditions for primer/probe concentrations may not be optimal when the targets are multiplexed. When building a multiplex panel, it is also advisable to add targets one at a time rather than combining all targets in the first experiment.

Using the same standard curve methodology, combine all primers and probes and perform the multiplex reaction for each dilution. Compare the resulting efficiencies for each target with their corresponding singleplex real-time PCR reaction efficiencies. Ideally, there will be little change between single and multiplex reaction standard curves. If the reaction efficiencies for the multiplexed targets vary by greater than 5% or fall outside the desirable range of 90–110%, optimization of the primer/probe concentrations or other components in the multiplex assay will be required.

The C_t values in the multiplexed reaction should be comparable to those obtained in the singleplex reactions so that the sensitivity is not compromised.

Troubleshooting

Introduction

Part of implementing an ideal real-time PCR assay involves optimization to ensure that all parameters of the reaction are finely tuned for accurate results. Assay validation accompanied by any required reaction adjustments should be performed any time a new gene is being studied or assay parameters are altered. This could involve adjusting primer concentrations, thermocycling temperatures and times, and then confirming the parameters through a standard curve assessment.

While optimization does take time, it is time well spent. The resulting assay will have the highest sensitivity and dynamic range, a high efficiency (which correlates with high accuracy), and excellent reproducibility. These factors all lead to confidence in the data and ultimately results accepted by the research community.

Troubleshooting a real-time PCR reaction can seem daunting. However, assuming proper assay design was taken into consideration, common real-time PCR difficulties can be grouped into four main areas:

- → Formation of primer-dimers
- → Storing primers and probes
- → Real-time PCR inhibition and poor reaction efficiency
- → Software analysis settings

Formation of primer-dimers

Primer-dimers form when partial sequence homology exists between the members of the primer pair. If the primers anneal to each other during the PCR reaction, the *Taq* DNA polymerase may be able to extend them and create a product larger than the original primers and one that is more prone to anneal erroneously as cycling progresses. Depending on its length, it is also possible for a primer to fold upon itself and therefore set up a competitive environment with the template. Reaction complexity, especially that present in multiplex reactions, increases the opportunity for these unwanted interactions to occur.

Primer-dimer formation is one of the most common problems you will have to troubleshoot during real-time PCR design and validation, but many opportunities exist to eliminate them from real-time PCR reactions. Before discussing how this is done, it is first important to understand why dimer formation should be minimized or reduced.

Problems caused by primer-dimers

The effect that primer-dimers can have on a reaction depends largely on the chemistry being employed. Fluorogenic probe–based reactions tend not to be influenced as much by primer-dimers because a probe annealing and being cleaved in a primer-dimer region is an extremely rare event. In this case, competition for primers is the main factor for consideration. Reactions that rely on double-stranded DNA–binding dyes, on the other hand, are highly

dependent upon primer-dimers being absent, because the dye would bind to them nonspecifically and therefore contribute to fluorescence signal being monitored during the reaction. This in turn shifts the C_t and skews results.

While the extraneous signal is the most important factor, competition within a reaction well also has a direct impact on reaction efficiency. As mentioned earlier, poor reaction efficiency shrinks dynamic range, which in turn decreases sensitivity.

It is best to take simple precautions during primer design to avoid dimerization in the first place. There are several free tools available to assist with this. One such tool is AutoDimer (authored by P.M. Vallone, National Institute of Standards and Technology, USA), which analyzes the sequences of primer pairs and flags those that, theoretically, have a tendency to dimerize.

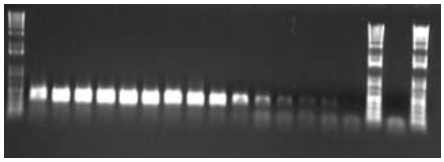


Figure 27—Agarose gel analysis to investigate primer-dimer formation. Prior to the thermal cycling reaction, the nucleic acid sample was serially diluted and added to the components of a PCR mix, and the same volume from each mixture was loaded on an agarose gel. Dimers appear as diffuse bands at the bottom of the gel.

Although bioinformatics-based primer design can reduce the likelihood of dimer formation, it is still necessary to monitor dimerization experimentally.

Determining if primer-dimers are present

Gel electrophoresis is a great way to visualize primer-dimers. As seen in Figure 27, primer-dimers appear as diffuse bands near the bottom of the gel, usually below 100 bp. During PCR there is competition between dimer formation and template annealing and extension. As template decreases, primer-dimers often increase.

The downside to gel analysis as the sole method of validation is that its sensitivity is in the low nanogram range and therefore may be inconclusive. The advantage of gel analysis is that the size of the product can help in the overall interpretation when dissociation curve (melt curve) data are also available.

Dissociation curves, also referred to as melt curves, are a standard part of reaction thermal profiles that employ double-stranded DNA–binding dyes. A very specific amplification will result in a single, tight peak on the dissociation curve for each well on the real-time PCR plate. Primer-dimers manifest themselves as lower-fluorescence, broader "waves" that indicate melting in the 70°C range. Peak shape and melting temperature is attributed to the small and variable sizes of primer-dimers. As mentioned in the data analysis section, if there is any doubt whether primer-dimers are present in a dissociation curve, compare the observation with the NTC well. A primer-dimer peak is much more common when template is absent (Figure 28).

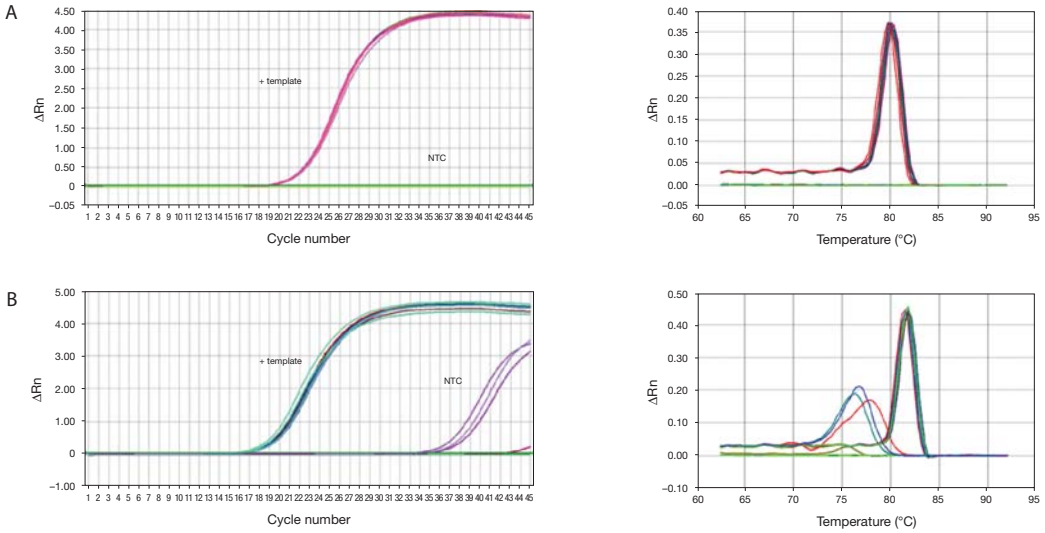


Figure 28—Amplification plots and melting profiles highlighting specific amplification (**A**) and primer-dimer effects (**B**). The primer-dimer can be identified in **(B)** by the signal produced by the NTC sample in the amplification plot, and additional peaks in the melting profile.

Reducing or removing primer-dimers

If primer-dimers are a concern, there are plenty of options to reduce or eliminate their occurrence in a reaction.

1. The first is optimization of the thermocycling conditions, which mainly involves raising the annealing temperature. In most cases, primers are designed so that they anneal successfully at 60°C, because two-step cycling (a 95°C denaturation step that goes directly to a 60°C annealing and extension step) facilitates robust amplification.
2. Primer concentration can always be lowered, and even employing different ratios of forward primer to reverse primer may help. In most cases, a final concentration of 200 nM per primer is ideal, but this can be reduced to 60 nM if necessary.
3. Magnesium is usually best at a concentration of about 3 mM. Primer-dimers are favored at concentrations above this.
4. If primers were not evaluated for their propensity toward dimerization, evaluate them and consider redesigning if necessary. And as usual, hot-start DNA polymerases and reaction setup on ice are also preferable.
5. Ideally, more than one primer set for the same target should be tested concurrently. This can actually save much time that would otherwise be spent on optimization if one of the pairs works immediately.

Keep in mind that dimers may be more of a concern in one-step qRT-PCR reactions due to the lower temperature of the RT reaction in the presence of the primer pair.

Storing primers and probes

Although often overlooked, primer and probe storage can have a major effect on the long-term success and consistency of a real-time PCR assay. The main factors that affect primer and probe stability are the storage temperature, length of time in storage, whether they have undergone prolonged exposure to light, the concentration of the stored primer or probe, and the composition of the storage solution.

Problems caused by poor storage of primers and probes

Improper storage of primers and probes can cause them to degrade and lose specificity, which in turn affects the reaction efficiency. In assays that rely on fluorescently labeled primers and probes, degraded probe releases free dye, which increases background and decreases the signal-to-noise ratio. This can manifest as very rough amplification curves due to the low fluorescence. Fluorescent dyes attached to primers and probes can also undergo photobleaching over time, making them less detectable in the real-time PCR instrument.

Determining if primer or probe integrity is compromised

The first preventive measure to ensure primer and probe stability is simple monitoring of the storage time. In many cases, primers and probes are stable for up to a year (or more). However, under suboptimal conditions, storage-related effects may be observed within 6 months.

The best method to evaluate primer integrity is consistent employment of standard curves. Replicate inaccuracy and multiple peaks in the dissociation curve, especially if not seen previously, are common signs that stability is low.

In the case of fluorescently labeled probes and primers, observing a higher-than-normal level of background fluorescence on the instrument's multicomponent view is indicative of probe degradation.

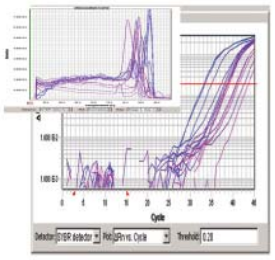
If the fluorescent probe or primer is not degraded but the dye itself is, an ethidium bromide–stained gel can show when product is made but not detected by the real-time PCR instrument.

Figure 29 shows standard curves highlighting the effects of poorly stored primers. The amplification plot in Figure 29A is negatively affected by degraded primers, while the curves in Figure 29B are what one would expect from properly stored primers. The melt curve inset provides additional detail, showing that multiple nonspecific products are present.

Maintaining primer and probe stability over time

There are four keys to maintaining primer and probe stability. Lyophilized primers have more flexibility with respect to storage time and temperature. Once reconstituted, primers should be kept at –20°C and should be monitored for signs of decreased functionality beyond a year or so. For labeled primers and probes, measures that protect the labels from light (such as the use of opaque tubes and dark storage) extend their life.

A



B

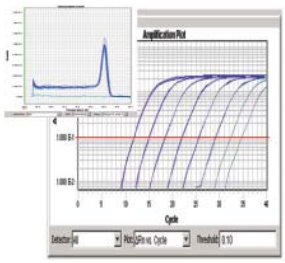


Figure 29—Amplification plots showing effects of poorly stored primers (shown by the melt curve and background fluorescence) (**A**) vs. properly stored primers (**B**).

Lastly, primer concentration can have an effect on stability. Storing primers at a concentration below 10 µM is not recommended; in fact, primer concentrations of 100 µM are easier to work with in most cases. Primers and probes should also be stored in aliquots to minimize freeze-thaw cycles, especially when labeled. Lastly, TE buffer creates a more stable environment than water. Moreover, TE that contains 0.1 mM EDTA (compared to 1 mM EDTA in standard TE) is a good choice because of the sensitivity of some PCR reactions to EDTA that may be carried over.

Real-time PCR inhibition and poor reaction efficiency

At this point, the importance of reaction efficiency should be well understood among the critical factors in real-time PCR assay design and optimization. To review, a standard curve (generated from a dilution series of the target template) is used to obtain an efficiency value. This efficiency value acts as a marker of overall reaction "health." Low efficiency leads to one diagnosis, high efficiency, another. Steps to improve these scenarios will be quite different. While the ideal reaction efficiency is 100%, the widely accepted range is 90–110%.

Causes of high or low efficiency

An efficiency above 110% indicates that **inhibition** is occurring in the reaction. Causes of inhibition include poor RNA or DNA quality, high template concentration, and carryover from nucleic acid purification. For example, if silica columns are employed, chaotropic salts used to bind the DNA or RNA might inhibit the *Taq* DNA polymerase. If organic extractions are used, phenol and ethanol carryover would have the same effect.

Inhibition is normally less common than **poor reaction efficiency**, which is an efficiency below 90%. Causes include suboptimal reagent concentrations (mainly primers, magnesium, and *Taq* DNA polymerase, especially for multiplex experiments). Other factors contributing to poor reaction efficiency include primer T_ms being more than 5°C different from each other and suboptimal thermocycling conditions. As mentioned earlier, competition for resources in the tube can produce an inefficient reaction.

Whether an efficiency for a target is high or low, matching efficiencies between a target and a normalizer is quite important for maintaining data accuracy. For example, an efficiency of 95% for target A and 96% for normalizer B is more desirable than an efficiency of 99% for target A and 92% for normalizer B.

The problem with skewed efficiency

Efficiencies outside the range of 90–110% may artificially skew results and lead to false conclusions, mainly because targets for comparison will have different efficiencies. In addition, inhibition and poor efficiency can affect assay sensitivity, leading to a smaller dynamic range and decreased versatility (Figure 30).

Determining if efficiency is skewed

As mentioned earlier, the best method for determining whether a particular assay is inefficient is to generate a standard curve of template diluted over the range of what will be encountered with the unknown samples and look at the efficiency over that range. It should be as close to 100% as possible.

A dissociation curve or gel showing multiple peaks or products means there is a competition for reaction resources that almost certainly will have an effect on the reaction efficiency.

Resolving poor efficiency or inhibition

Once it has been determined that the reaction is inhibited or is operating with poor efficiency, there are some steps that can be taken to bring the efficiency value back into the desirable range.

1. For inhibition, those wells with the highest concentration of template can be removed and the standard curve reanalyzed. If the efficiency improves back to under 110%, the assay is fine. Just keep in mind that any concentrations removed from the standard curve may not be used during the actual assay.
2. Another solution involves re-purifying the template. Remember to allow extra drying time to remove ethanol from ethanol precipitations or to employ additional on-column washes to remove chaotropic salts from silica-based purifications.
3. Poor efficiency is resolved through assay optimization. Sometimes the process can be relatively pain-free, but in other situations, as assay complexity increases, optimization can be laborious.
   a. Raising the magnesium concentration as high as 6 mM can improve efficiency in situations where a single product is amplified, but

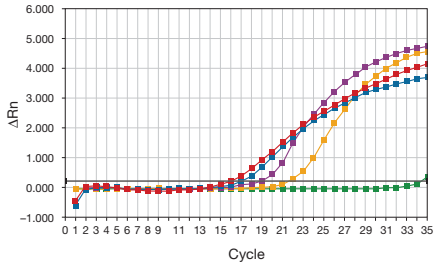


Figure 30—Template dilution series to assess reaction efficiency. Dilutions with earlier C_ts exhibit compressed C_ts and abnormal curve shapes. As the template becomes more dilute, inhibition vanishes and the curves take on the more characteristic exponential phase shape.

lowering the magnesium may help in cases where competition is occurring.

   b. In some circumstances, mainly multiplexing reactions, a primer and probe optimization matrix is necessary. In this application, different ratios or concentrations of forward primer to reverse primer, and sometimes even probe ratios, are tested to find the ideal concentration combination for a given assay. The ideal primer concentration can be anywhere from 100 to 600 nM, while probe concentrations can be between 100 nM and 400 nM.
   c. Ensure that the thermocycling conditions (especially the annealing temperature) are favorable based on the T_ms of the primers, and that the primers are designed to have similar T_ms.

In some cases, issues that appear to be reaction-related may in fact be software-related. Validating and/or optimizing software settings can often bring results back in line with expectations.

Software analysis settings

As mentioned, there are cases in which the instrument analysis may be masking an otherwise successful assay. Analysis settings that play the largest role in data accuracy are:

→ Amplification curve baseline linearity
→ Baseline range settings
→ Threshold
→ Employing reference dyes

The **amplification curve baseline linearity** is one parameter that can affect results. The instrument software is usually good at automatically setting the baseline within the flat portion of the curve. However, in cases where a very early C_t is observed, such as in cases where 18S is used as a normalizer, the baseline can mistakenly be placed to include a region that is no longer flat. Figure 31 shows the same plot with different baseline settings. Figure 31A shows a plot with a baseline that spans cycles 1 through 14, which is too wide because fluorescence is detected as early as cycle 10. The result is a curve that dips down and pushes the C_t later. Figure 31B shows the baseline reset to the linear range of cycles 2 through 8 and returns the curve and C_t to their accurate locations.

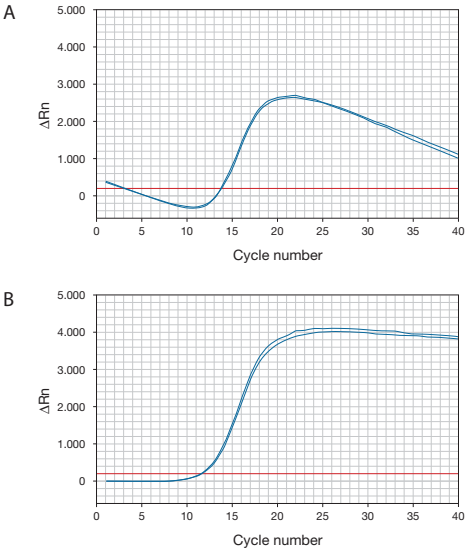


Figure 31—Amplification plots showing an incorrectly set baseline (**A**) and a correctly set baseline (**B**).

Baseline range settings are not often considered but can have an effect on the reaction efficiency. In Figure 32, starting on the left, the baseline is manually set very wide and the slope of the standard curve is poor, only –2.81, which is well outside the preferred range of –3.58 to –3.10 (corresponding to 90–110% efficiency). The plots on the right are the same curves but with a baseline that the instrument automatically chose. The slope is now inside the ideal window and the assay is now validated across this dynamic range. However, the plots in the middle were manually adjusted to have the baseline incorporate 4 to 5 additional cycles, and the slope improved even further to nearly 100% efficiency. This indicates that the instrument default settings are more often than not acceptable for a given assay. However, manual adjustment can sometimes improve results even further.

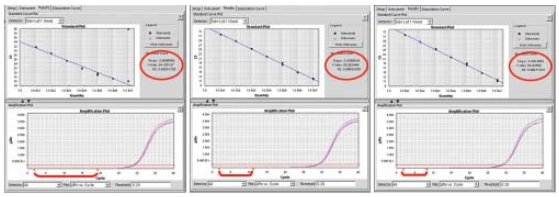


Figure 32—Comparison of baseline setting methods to achieve acceptable reaction efficiencies.

The **threshold** (the level of fluorescence deemed above background and used to determine C_t) is another parameter set automatically by the software, but one that may also be manually adjusted. The most accurate portion of an amplification curve for C_t determination is directly in the middle of the exponential phase when viewing the log plot. The ideal threshold setting is sometimes unique for each set of data (Figure 33).

Again, while the default settings are often very appropriate, the threshold can be manually dragged to the middle of the exponential phase for greater accuracy if needed.

Standard curve dynamic range validation determines what template concentrations are acceptable in a given assay. Concentrations from the high and/or low ends of a standard curve can also be removed to improve the efficiency of a reaction, as long as those concentrations are never employed during the actual assay (Figure 34).

Employing reference dyes such as ROX and fluorescein is a powerful method of insulating against some instrument- and user-related error. It has become so common that this "behind-the-scenes" factor is often forgotten or assumed not to have a negative impact on a reaction. However, it is important to understand the relationship between the instrument software and the dye itself.

For instruments that employ a reference dye, the software subtracts the inert dye's fluorescence signal from the fluorescence emitted by the target. Therefore, if the level of ROX, for example, is too high, it can result in very poor target signal returns, which manifest as jagged and inconsistent amplification plots.

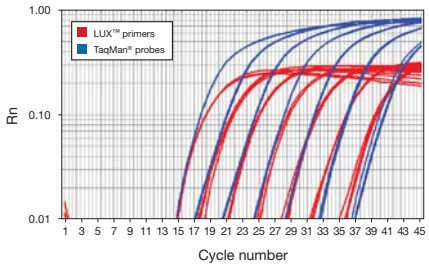


Figure 33—Log plot screen shot showing an example of two sets of curves with differing plateau heights and therefore different exponential phases. When evaluating more than one kit or chemistry on the same run, this situation can often occur. The software will automatically select a threshold that is ideal for the curves with the higher plateau (the blue plots in Figure 35B). This would bias the C_ts for the red plots in that data set because the ideal threshold is much lower. Therefore, each data set should be studied independently so that the ideal threshold may be selected for each situation.

The amplification plot in Figure 35 illustrates this finding:

Visually, it seems as if the reaction failed and much optimization is necessary, but where to start? If the ROX channel is switched off as the normalizer and data are reanalyzed, it can be seen that the data are actually just fine (Figure 35B); it is a reference dye normalization issue.

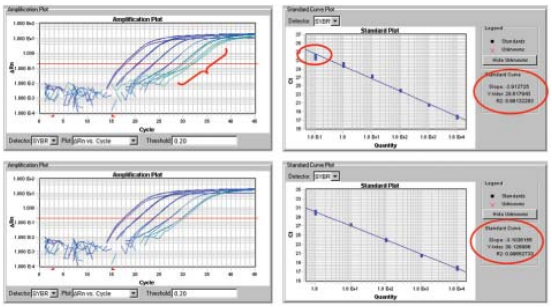


Figure 34—Improvement of standard curve slope achieved by excluding outlier data. Amplification was performed on a dilution series over 5 orders of magnitude. Across this range the slope is only –2.9, which is outside the desired efficiency window. The curves represented by the lower panels have had the highest dilutions omitted from the standard curve, and the efficiency reanalyzed. The slope has improved to –3.1, which is considered efficient enough to validate the assay. In general, it is fine to push the limits of detection from very high template to very low template, knowing that terminal data points can always be removed if efficiency is compromised.

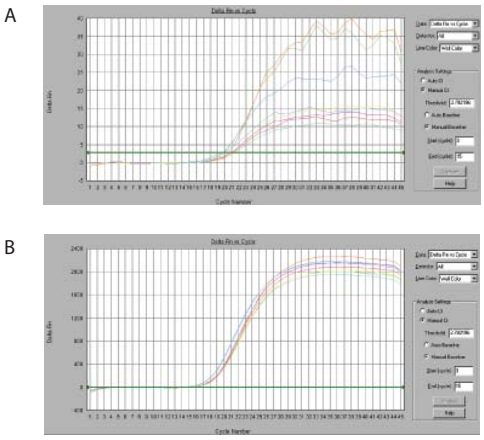


Figure 35—Correction for reference dye. **A.** A high level of a passive reference dye such as ROX can lead to poor target signal returns. **B.** Once the signal from ROX is removed from the analysis, the target signals fall within the expected range.

As mentioned, the default instrument software settings are fine in most situations. However, verification of these settings can increase confidence in data accuracy. Ensure that the baseline chosen by the software is only in the flat range of the amplification curve, and increase or decrease the baseline range as necessary. Look at the amplification curves in the log plot view and verify that the threshold is set near the middle of the exponential phase of the curve. Adjust the y-axis to be appropriately scaled for the fluorescence plateau intensity, and keep in mind that outliers and whole dilution sets may be removed from the standard curve to improve efficiency and R^2 values (as long as those dilutions will not be used when evaluating the "unknown" samples). Lastly, keep in mind that baseline and threshold settings need to be identical between any assays that will be compared.

Troubleshooting is an inevitable aspect of real-time PCR assay validation and employment. However, by categorizing and understanding the key issues, this can be a relatively painless process. Ensure that primer-dimers are not contributing to signal or poor reaction efficiency. Take the steps necessary to maintain primer and probe stability. Make standard curve validation the final step in the reaction assessment process. Understand that efficiencies below 90% will be addressed very differently from values above 110%. Lastly, verify and adjust instrument analysis settings as necessary.

Frequently asked questions

Q: How many copies are in a given amount of human genomic DNA?

A: 1 genome copy = 3 x 10^9 bp
1 bp = 618 g/mol
1 genome copy = (3 x 10^9 bp) x (618 g/mol/bp)
= 1.85 x 10^{12} g/mol
= (1.85 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} [Avogadro's number])
= 3.08 x 10^{-12} g
Each somatic cell has 6.16 pg of DNA (sperm and egg cells have 3.08 pg). There is one copy of every nonrepeated sequence per 3.08 pg of human DNA. Therefore, 100 ng of genomic DNA would have: (100,000 pg of DNA)/3.08 pg = ~33,000 copies; 1 ng of DNA has 330 copies.

Q: Why do I have to be concerned about the efficiency of my qPCR?

A: If you want to compare the expression levels of two genes (for example, in cases where a normalizer gene is employed), you need to know something about the efficiencies of the PCR to confirm that the C_t values you are observing are not being influenced by contaminants in the PCR reagents or are not arising from a poorly optimized assay.

Q: I have found that my more concentrated template samples give me less efficient amplification curves:
Dilute sample gives a slope of –3.4 and an R^2 value of 0.99; concentrated sample gives a slope of –2.5 and an R^2 value 0.84.

A: Something in your sample is inhibiting the PCR, sometimes. The reason you get better efficiency with the more diluted samples is because the inhibitor (salt or some other component) has been diluted below its inhibitory effect. Here are some references which explain this:

Ramakers, C. et al. (2003) Assumption-free analysis of quantitative real-time Polymerase Chain Reaction (PCR) data. *Neurosci Lett* 339:62–66.

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

Bar, T. et al. (2003) Kinetic Outlier Detection (KOD) in real-time PCR. *Nucleic Acids Res* 31:e105.

Q: Can I compare C_t values of PCR reactions with different efficiencies?

A: You should not compare C_t values of PCR reactions with different efficiencies, because the $\Delta\Delta C_t$ calculation method works on the assumption that PCR efficiencies are comparable. This is why you should optimize your system before trying to quantify unknown samples. The standard curve method of comparative quantification with efficiency correction can be employed.

Q: Why is a reference dye used with LUX™ primers in qPCR?

A: It is very useful to normalize fluctuations in fluorescence resulting from real-time PCR instrument noise, especially if you are detecting low copy numbers. Depending on your instrument, the reference dye may be ROX or fluorescein. The main purpose is to detect changes in fluorescence that are not related to the accumulating PCR product.

Q: Why can't I use the Bio-Rad iCycler® instrument to perform multiplex real-time PCR with FAM- and JOE-labeled LUX™ primers?

A: Because of the filter/channel setup, the Bio-Rad iCycler® instrument can't distinguish FAM and JOE. These two dyes' wavelengths fall into the same channel on the iCycler® instrument, so they will be read as one emission.

Q: What are quenchers, and why are they used in real-time PCR? Are quenchers required when using LUX™ primers?

A: Quenchers are moieties attached to primers or probes so that they can quench the emission from a fluorophore that is also attached to that primer or probe. Quenchers are generally used in probe-based assays to extinguish or change the wavelength of the fluorescence emitted by the fluorophore when both are attached to the same oligo. They usually do this by FRET (fluorescence resonance energy transfer). When the fluorophore gets excited it passes on the energy to the quencher, which emits the light at a different (higher) wavelength. Common quenchers are Dabcyl, TAMRA, and Dark or Black Hole Quencher® dyes. LUX™ primers do not need a quencher because they form a hairpin loop that quenches the unextended primer.

Q: **When would I use one-step as opposed to two-step qRT-PCR ?**

A: Two-step qRT-PCR is popular and useful for detecting multiple messages from a single RNA sample. It also allows the archiving of cDNA for further analysis. However, one-step qRT-PCR is easier to use when processing large numbers of samples and helps minimize carryover contamination, since tubes do not need to be opened between cDNA synthesis and amplification. Since the entire cDNA sample is amplified, one-step qRT-PCR can provide greater sensitivity, down to 0.1 pg total RNA.

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Further reading

A-Z of Quantitative PCR (S.A. Bustin, editor) International University Line (IUL), La Jolla, California, USA

Appendix

$$N = \frac{C}{K} 182.5 \times 10^{13}$$

N = Molecules per µl
C = cRNA concentration (µg/µl)
K = Fragment size (nt)

1. The above equation was reproduced from Fronhoffs, S. et al. (2002) *Mol Cell Probes* 16:99–110. The 182.5 x 10^{13} value is described as a factor derived from the molecular mass and Avogadro's constant.
2. From Bustin's *A-Z of Quantitative PCR*, we can also consider a formula that is universal for all RNAs.
   a. Calculate RNA molecular weight (MW) from the number of nucleotides of each type in the sequence :
      MW = (#A x 328.2) + (#C x 304.2) + (#G x 344.2) + (#U x 305.2)
      Result is expressed in g/mol.
   b. Calculate number of molecules per ng using Avogadro's number:
      (1 x 10^{-9} g/MW (from a. above)) x 6.02 x 10^{23} molecules per mole.
   c. Calculate number of molecules per µl:
      (molecules per ng (from b. above)) x (concentration in ng/µl)

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