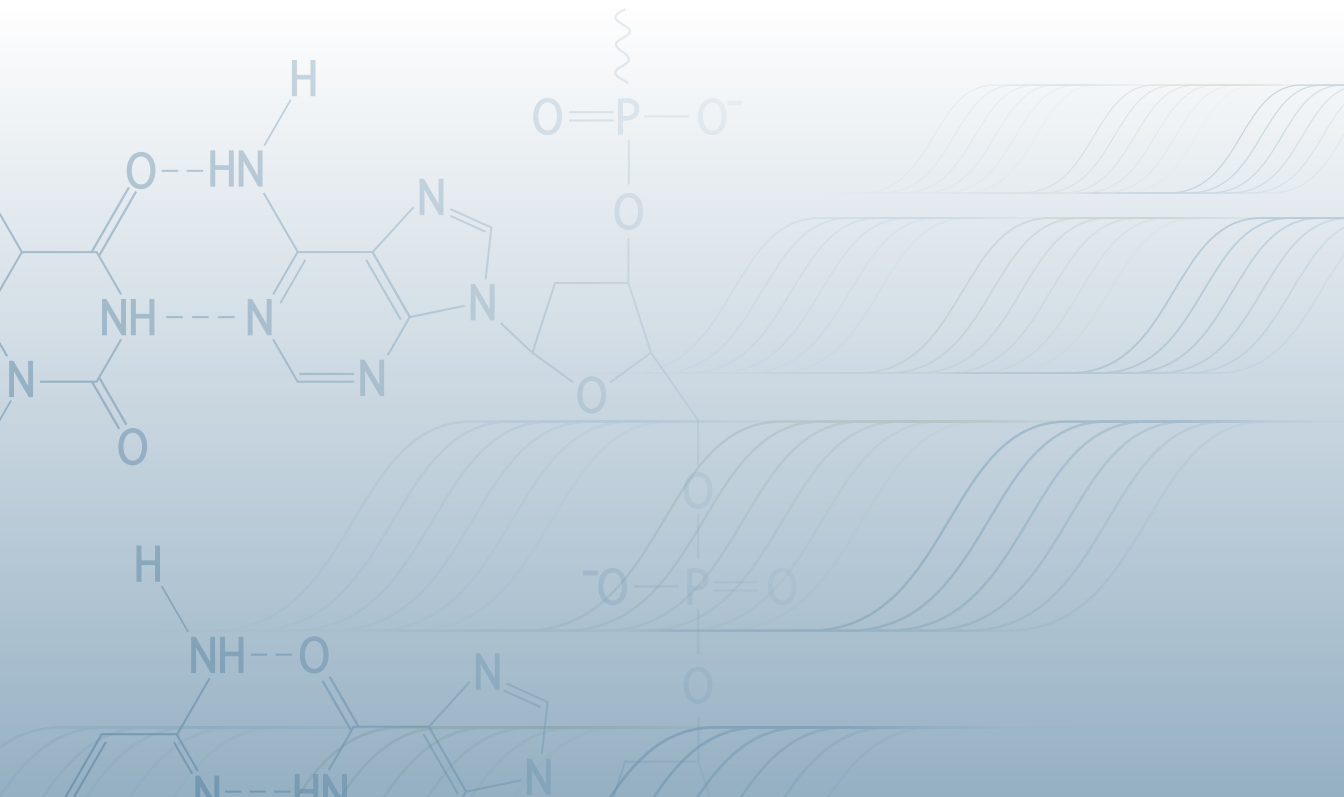
PrimeTime®

INTEGRATED DNA TECHNOLOGIES

qPCR Application Guide

Experimental Overview, Protocol, Troubleshooting

Third Edition



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Third Edition

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1. Introduction

This *qPCR Application Guide* is intended to provide guidance on the entire qPCR process, from RNA isolation to data analysis. This document should be used to obtain a basic understanding of everything involved in the experimental setup, performance, and analysis. The guide begins with a general overview of qPCR and then provides more specific information on design, experimental setup, data analysis, and troubleshooting for the 5′ nuclease assay. A general protocol for using PrimeTime® qPCR Assays for this process is included. Importantly, this document follows the recommendations provided in *The MIQE guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments* and two recent updates [1], which are a definitive guide and excellent resource for all of the necessary requirements for experimental setup, analysis, and publication.

1.1 Advantages of qPCR

Quantitative real-time PCR (qPCR) has become the most precise and accurate method for analyzing gene expression. Prior to qPCR, the most common methods for determining expression levels were northern blotting, RNase protection assays, or traditional, endpoint reverse transcription (RT) PCR. Endpoint RT-PCR was an improvement over the older methods due to its ease of use and the much smaller amounts of RNA needed for the reaction. However, with this method, expression levels can only be observed by performing agarose gel electrophoresis on a sample of the product at the end of the entire reaction. While traditional RT-PCR can be useful for determining the presence or absence of a particular gene product, qPCR has the advantage of measuring the starting copy number and detecting small differences in expression levels between samples. qPCR allows investigators to observe PCR product accumulating over the entire amplification curve and eliminates the need to run a gel, which reduces the duration of the process and the chance of contamination. Amplification and quantification occur simultaneously. A typical qPCR amplification plot has baseline, exponential, linear, and plateau phases (Figure 1). Amplification reaches a plateau as the reaction components are exhausted and PCR products self-anneal and thus prevent further amplification. In endpoint PCR, amplification can only be viewed at the end of the reaction, and only the final plateau is observed—any differences in initial abundance are obscured. In contrast, qPCR quantifies the PCR products while the amplification is in progress. Fluorescent reagents allow amplification to be measured while the reaction is occurring through use of a fluorescence detector in conjunction with the thermal cycler. This allows analysis of the entire amplification curve rather than only the end point.

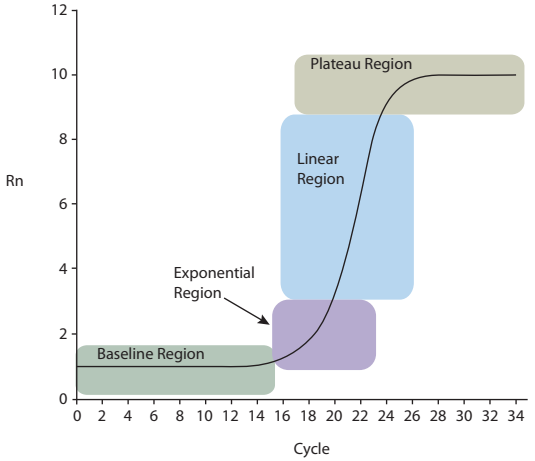


Figure 1. Phases of a Typical qPCR Amplification Plot. qPCR amplification plot phases include baseline, exponential, linear, and plateau regions of the amplification curve.

1.2.1 5′ Nuclease Assay

The 5′ nuclease chemistry utilizes two primers and a hydrolysis probe, and capitalizes on the exonuclease activity of Taq DNA polymerase [3].

- The DNA probe is non-extendable and labeled with a fluorescent reporter dye (D) and 1 or 2 quenchers (Q), which are maintained in close proximity to each other while the probe is intact (Figure 2). The presence of one of the quenchers at the 3′ end prevents extension of the probe by the polymerase.
- While the probe is intact, the energy emitted by the reporter dye is absorbed by the quencher(s).
- Because all three components (2 primers and 1 probe) must hybridize to the target, this method allows detection of the PCR product with greater specificity and higher accuracy. In addition, different probes can be labeled with different fluorescent dyes, enabling multiple targets to be simultaneously detected in a single reaction [4,5]. See Section 4.2.4 for more information on multiplex reactions.
- When the primers and probe hybridize to the target and the polymerase begins to extend the primers, the probe is hydrolyzed by the 5′ to 3′ exonuclease activity of the polymerase causing the reporter and quencher(s) to dissociate from the target. The spatial separation of reporter and quencher(s) disrupts the ability of the quencher(s) to absorb energy emitted from the reporter and, thus, a substantial increase in re-

porter dye fluorescence occurs [6]. The fluorescence produced during each cycle is measured during the extension phase of the PCR.

- Examples of 5′ nuclease assays include PrimeTime® qPCR Assays (IDT) and TaqMan® Assays (Applied Biosystems). (See Section 4.1 for more information about PrimeTime qPCR Assays and ZEN™ double-quenched probes.)

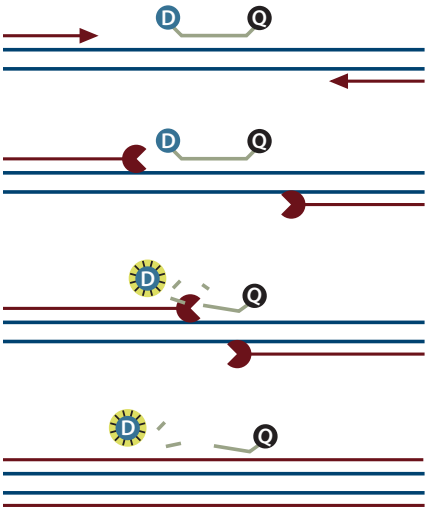


Figure 2. 5′ Nuclease Assay. During the annealing step, the primers and probe hybridize to the complementary DNA strand in a sequence-dependent manner. Because the probe is intact, the fluorescent reporter (D) and quencher (Q) are in close proximity and the quencher absorbs fluorescence emitted. In the extension step, the polymerase begins DNA synthesis, extending from the 3′ ends of the primers. When the polymerase reaches the probe, the exonuclease activity of the polymerase cleaves the hybridized probe. As a result of cleavage, the fluorescent dye is separated from the quencher and the quencher no longer absorbs the fluorescence emitted by the dye. This fluorescence is detected by the real-time PCR instrument. Meanwhile, the polymerase continues extension of the primers to finish synthesis of the DNA strand.

1.2.2 Molecular Beacons

- Molecular beacons are labeled with a 5′ fluorescent reporter dye (D) and a 3′ quencher (Q). A stem region is designed such that, at annealing temperature and in the absence of target, the ends of the beacon are held closely together, which allows the quencher to absorb the fluorescence from the reporter (Figure 3).
- When the probe hybridizes to its target sequence during the annealing phase of the PCR, the quencher is no longer in close proximity to the fluorophore dye and the reporter fluoresces [8]. The resulting fluorescence is measured during this annealing phase.
- The uncleaved probe dissociates during the extension step and can participate in the next PCR cycle.
- Molecular beacons will thermodynamically favor the hairpin structure over a non-specific target sequence, which makes these probes highly specific. A perfect match probe–target hybrid will be energetically more stable than the stem-loop structure, whereas a mismatched probe–target hybrid will be energetically less stable than the stem-loop structure [9]. IDT synthesizes the probes and primers needed for this system.

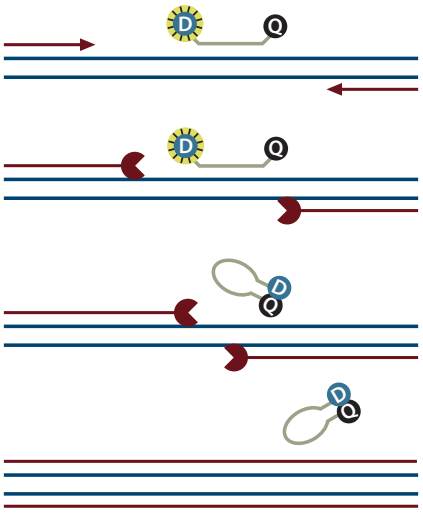


Figure 3. Molecular Beacons. During the annealing step, the primers and molecular beacon probe hybridize in a sequence-dependent manner to the target DNA. Hybridization of molecular beacons to the complementary target separates the fluorescent dye (D) and quencher (Q) so that the quencher no longer absorbs the energy emitted by the fluorophore. The resulting fluorescence is detected by the real-time PCR instrument. Molecular beacons in excess of the target DNA sequence reform the hairpin during the annealing phase and the quencher absorbs the fluorescence , emitting the energy as heat. In the extension step, the polymerase begins DNA synthesis, extending from the 3′ ends of the primers. When the polymerase reaches the molecular beacon, the molecular beacon is displaced without being degraded. Thus probe can participate in multiple rounds of annealing. Meanwhile, the polymerase continues extension of the primers to complete synthesis of the DNA strand.

1.2.3 Hybridization/FRET Probes

Hybridization/FRET probes consist of two separate fluorescent dye–labeled probes; one probe is labeled on the 3′ end with a donor fluorophore (D) and the other is labeled on the 5′ end with an acceptor fluorophore (A) (Figure 4).

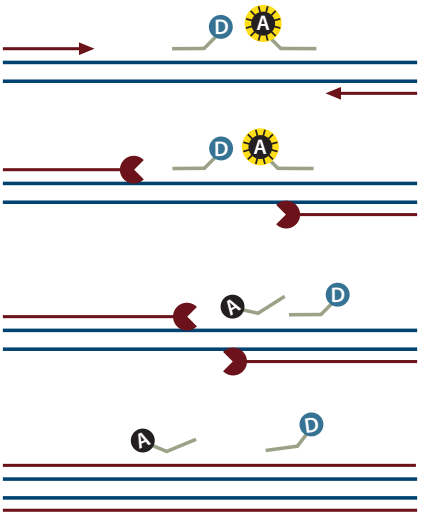


Figure 4. Hybridization/FRET Probes. During the annealing step, the primers and probes hybridize in a sequence-dependent manner to the complementary DNA sequence. The probe containing the activated 3′ donor fluorophore (D) is positioned to transfer its energy to a second probe containing a 5′ acceptor fluorophore (A). The fluorescence of the acceptor is recorded by the instrument. In the extension step, the polymerase synthesizes new DNA strands by extending from the 3′ ends of the primers.

- The 3′ end of the acceptor probe contains a phosphorylation modification that prevents extension of the probe during amplification. Since the 3′ end of the donor probe is labeled with a fluorophore, no phosphorylation is required to block extension on this probe.
- During the annealing step, the primers and both probes hybridize to the target with the probes in a head-to-tail configuration, which brings the two fluorophores close together.
- A light source is used to excite the donor fluorophore which then excites the acceptor reporter fluorophore through FRET (Fluorescence Resonance Energy Transfer). A detector is set to read the emission wavelength of the acceptor fluorophore.
- In order for the energy transfer to occur, the spectra of the two fluorophores must overlap so that the donor fluorophore can excite the acceptor fluorophore.
- These types of probes require dedicated machinery in order to excite the donor fluorophore. The LightCycler® Real-Time PCR System (Roche) is designed for such probes. IDT synthesizes the probes and primers needed for this system.

1.2.4 Scorpions™ Probes

Scorpions™ probes consist of a primer covalently linked to a spacer sequence followed by a probe that contains a fluorophore and a quencher (Figure 5).

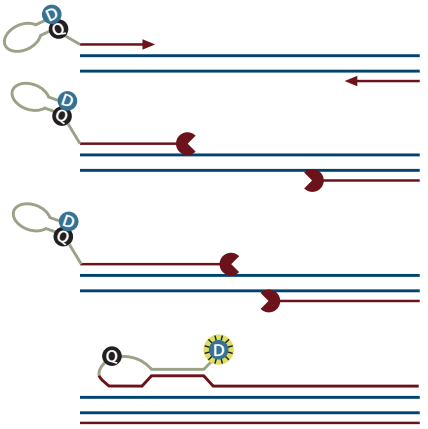


Figure 5. Scorpions™ Probes. During the annealing step, the primers hybridize in a sequence-dependent manner to the complementary DNA strand. The probe, attached to one of the primers, remains in a hairpin structure with the fluorescent dye (D) and quencher (Q) in close proximity, the quencher absorbing the fluorescence energy emitted by the dye. In the extension step, the polymerase begins DNA synthesis, extending from the 3' ends of the primers. As extension continues and the complementary sequence is synthesized, the loop sequence of the probe hybridizes to the complementary target sequence of the newly synthesized strand. This separates the probe dye and quencher so that the quencher no longer absorbs the energy emitted by the dye. The fluorescence is detected by the real-time PCR instrument.

- The probe contains an amplicon-specific, complementary target sequence in the loop portion of the stem-loop, a spacer sequence, a fluorophore dye (D), and an internal quencher (Q), all contiguous with the primer.
- When not bound to the target, the probe remains in a stem-loop structure, which keeps the quencher and fluorophore dye proximal and allows the quencher to absorb the fluorescence energy emitted by the dye.
- During PCR, the primer binds to the target for the first round of target synthesis. Because the primer and probe are connected, the probe becomes attached to the newly synthesized target region. The spacer region prevents the DNA polymerase from replicating the probe sequence, disrupting the stem structure and rendering the amplicon permanently fluorescent.
- When the second cycle begins, binding of the loop sequence to the amplicon is thermodynamically favored over binding to the hairpin stem. The probe is denatured and hybridizes to the target, separating the fluorophore and quencher. The resulting fluorescence emission can be detected during the annealing phase.

1.2.5 Intercalating Dyes

Intercalating dyes are nonsequence-specific fluorescent dyes that exhibit a large increase in fluorescence emission when they intercalate into double-stranded DNA. Examples include SYBR® Green I, Cyto, EvaGreen®, and LC dyes [10,11]. During PCR, the primers amplify the target sequence and multiple molecules of the dye are inserted between bases of the double-stranded product, causing fluorescence (Figure 6).

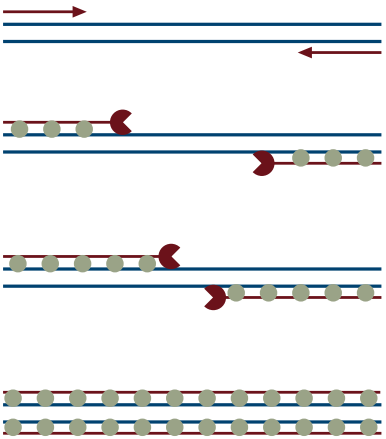


Figure 6. Intercalating Dye. During the annealing step, the primers hybridize in a sequence-dependent manner to the complementary DNA strand. In the extension step, the polymerase begins DNA synthesis, extending from the 3′ ends of the primers. As the amplicon is extended, the intercalating dye binds to the newly formed double-stranded DNA. Fluorescence increases in a sequence-independent manner with increasing amplicon length. When DNA synthesis is completed, the final amount of fluorescence, a function of both the length and number of copies of the amplicon, is determined. Finally, during denaturation, the dyes dissociate from the amplicon.

- Intercalating fluorescent dyes are not specific to a particular sequence; thus, they are both inexpensive and versatile.
- As they can bind to any double-stranded sequence, they will also bind to primer-dimer artifacts or incorrect amplification products [12]. Therefore, when using intercalating dyes, it is important to analyze the melting curve of the amplicon to ensure that the primers are amplifying a single product, observed as a single melting curve peak.
- These types of dyes cannot be used for multiplex analyses as the different PCR products would be indistinguishable. See Section 4.2.4 for more information on multiplex reactions.
- Because multiple dye molecules intercalate into a double-stranded product, the intensity of the fluorescent signal is dependent on the mass of the amplified product. Assuming both amplify with the same efficiency, a longer product will generate more signal than a shorter product [8]. In contrast, probes are specific to a particular sequence and will emit the same amount of energy from a single fluorophore irrespective of the length of the amplified product, creating a 1:1 ratio between the fluorescence emission of a cleaved probe and recognition of one amplicon molecule.

Related Products at IDT

IDT offers PrimeTime® qPCR products for the 5' nuclease assay. PrimeTime qPCR Assays consist of a forward primer, a reverse primer, and a labeled probe, all delivered in a single tube. The PrimeTime qPCR probe is a non-extendable oligonucleotide that is labeled with a fluorescent reporter and a quencher dye. PrimeTime Predesigned qPCR Assays are available in three different scales and five different dye–quencher combinations for human, mouse, and rat transcriptomes. PrimeTime Predesigned qPCR Assays are prepared when ordered using up-to-date sequence information from NCBI, and are guaranteed to be at least 90% efficient when used with a commercially available master mix and measured over 4 orders of magnitude. IDT offers PrimeTime qPCR Primers that are ideal for SYBR® Green or other intercalating dye assays, where no probe is needed. These predesigned primer pairs are identical to those in PrimeTime Predesigned qPCR Assays. For increased sensitivity and decreased background, IDT also offers assays and probes with the internal ZEN™ Quencher (Double-Quenched Probes), available with FAM, HEX, and TET dyes. Custom primers, molecular beacons, and hybridization/FRET probes can all be obtained from IDT.

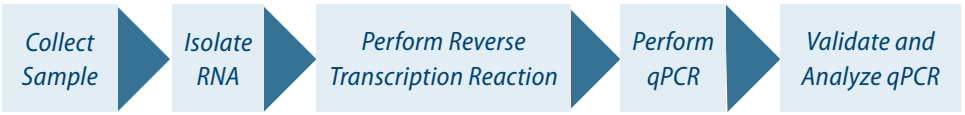
In addition, IDT offers SciTools® Design Tools, a suite of free design and analysis tools that include the PrimeTime qPCR Assay Design Tool for identifying current PrimeTime Predesigned qPCR Assays, the RealTime PCR and PrimerQuest℠ design tools (for designing primers, probes, and assays), OligoAnalyzer® program (for analyzing oligonucleotide melting temperature, hairpins, dimers, and mismatches), and UNAFold program (for analysis of oligonucleotide secondary structure).

For more information, to order any of these products, or to use the free design tools, visit the IDT website at www.idtdna.com.

1.3 qPCR Workflow

The typical qPCR experiment involves the following steps:

- Sample collection.
- RNA isolation and quality control (Section 2).
- Reverse transcription (Section 3).
- Real-time PCR (Section 4).
- Assay validation and data analysis (Sections 5 and 6).



Each of these steps is covered in this guide along with recommendations for proper experimental setup and design. All of the recommendations follow the MIQE guidelines (See Section 8.1 and reference 1].

2. RNA Isolation and Quality Control

The first steps in running a qPCR assay are to collect the sample and isolate total RNA. The method of RNA isolation will depend on the sample type and experimental conditions. After isolation, both the quantity and quality of the RNA should be assessed. Throughout the process, it is imperative that the sample remain free of RNases and DNases. Here, we provide some suggestions for isolation, quantification, assessing quality, and preventing nuclease contamination.

2.1 Isolate

RNA isolation can be performed using organic extraction methods (TRIzol® reagent [Invitrogen], QIAzol® reagent [Qiagen], RNA STAT-60 [Tel-Test, Inc.], guanidium salt–based methods [2]), or a variety of solid phase RNA isolation kits that are available commercially from companies including Qiagen, Life Technologies (Ambion), and Promega. The best method will depend on your sample type and the amount of RNA available for harvesting. For example, small RNAs and miRNAs can only be efficiently isolated using organic extraction methods [13], while solid phase kits are the appropriate choice for high-throughput processing. It is important that the RNA be extracted from all samples using the same method, and that the resulting RNA be of high quality. Differences in either sampling or isolation methods can lead to unwanted variation between samples. Surfaces and supplies should be free of RNases. Some samples may require DNase treatment to remove genomic DNA contamination. This step may not be necessary if the assay is designed to span exon junctions, and thus, only contain only exonic sequences. If the isolated RNA is not going to be used immediately, it should be frozen at –20°C for short-term storage for, at most, a few months. For longer term storage, freeze and store the RNA at –80°C, or precipitate the RNA and store it in ethanol at –20°C.

2.2 Quantify

RNA can be quantified by several methods, including UV spectrophotometry, microfluidic analysis (capillary gel electrophoresis), or by the use of fluorescently-labeled RNA binding dyes [1]. Absorbance measurements at 260 nm on standard spectrophotometers can be used for quantification when RNA is abundant, while the NanoDrop (Thermo Scientific) and DropSense (Trinean) instruments are useful for measuring limited quantities of sample. Microfluidic quantification can be performed using the 2100 Bioanalyzer (Agilent Technologies) or the Experion system (Bio-Rad Laboratories). These microfluidic

methods also enable RNA quantification of limited amounts of sample. In addition, microfluidic analysis enables simultaneous integrity assessment (see Section 2.3, below).

2.3 Check Quality

In addition to having similar quantities of RNA, it is also important that the samples be of similar quality. The quality of the RNA can have a large impact on the results of the experiment; poor quality RNA can compromise the entire experiment and result in wasted time and money. Furthermore, differences in quality between two samples can lead to misinterpretation of gene expression differences.

RNA quality can be assessed most accurately by calculating the integrity of the RNA. The Agilent 2100 Bioanalyzer (Agilent Technologies) or the Experion system (Bio-Rad Laboratories) can be used for this purpose. These instruments enable electrophoretic separation of very small amounts of RNA sample, which can be detected by laser-induced fluorescence. High-quality eukaryotic RNA will have both 18S and 28S rRNA peaks, with the 28S region in greater abundance, and a low amount of 5S RNA [14] (Figure 7). The RNA integrity value is determined from the shape of the resulting electropherogram curve and is based on several characteristics. The software uses an algorithm to assign a number to the RNA with 1 being the most degraded and 10 being the most intact [14]. The ideal integrity value will depend on the RNA source, as some tissues will provide higher quality RNA. See the publication from Fleige and Pfaffl [14] for more information on average integrity values for various tissues.

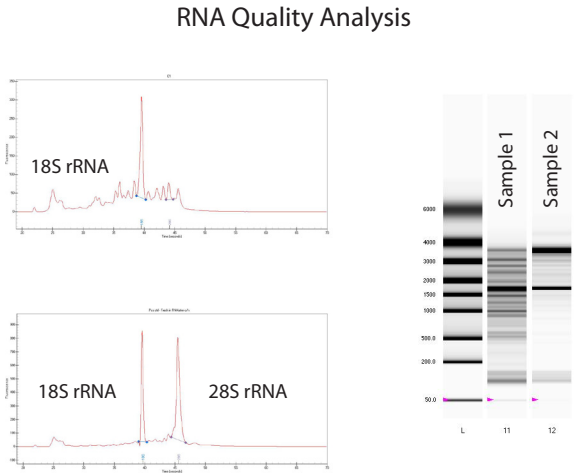


Figure 7. Examples of Sample Quality. Experion™ (BioRad) microfluidics analysis of poor quality RNA (Sample 1), and good quality RNA (Sample 2). The 28S rRNA species is more susceptible to degradation.

The ratio of 260/280 nm absorbance readings can also give an indication of RNA quality. However, other contaminants may affect this ratio, making it less accurate than analysis of the RNA integrity value. A ratio of 1.8 indicates the RNA is of good quality. Lower ratios could be due to organic compound contamination. Turbidity or low pH can also lead to calculation errors. To correct for the effects of turbidity when estimating RNA quality at neutral pH, readings at 320 nm should be subtracted from readings at 240, 260, and 280 nm. Alternative methods for determining quality include gel electrophoresis, microfluidics-based rRNA analysis, or a reference gene/target gene 3:5 integrity assay [1,15].

2.4 Avoid RNases, DNases

RNases and DNases are nucleases that can quickly degrade samples and oligonucleotide primers and probes. They are ubiquitous and can be difficult to eliminate. Therefore, it is very important to take precautions to ensure that samples are protected from degradation by these nucleases. Follow clean PCR guidelines (see next page) to prevent contamination and test the samples using nuclease detection reagents such as RNaseAlert™ and DNaseAlert™ Kits (IDT). If you do find contamination in your samples, be sure to replace all reagents and stock buffers and thoroughly clean the PCR preparative areas. RNase inhibitors can be added to block the action of some ribonucleases, and DNases can be inactivated by heat treatment.

Related Products at IDT

RNaseAlert™ and DNaseAlert™ Kits: These reagents are fluorescence-quenched oligonucleotide probes that emit a fluorescent signal only after nuclease degradation and allow for rapid, sensitive detection of RNases or DNases.

ReadyMade™ Primers and Randomers: IDT offers a number of primers and randomers, including random hexamers and Oligo(dT) primers, that are pre-made, purified, and ready to ship upon order.

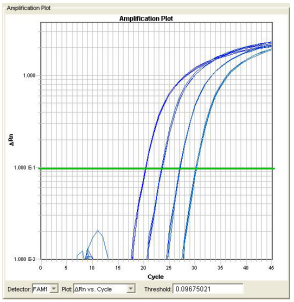
For more information and to order these products, visit the IDT website at www.idtdna.com.

Guidelines for Maintaining a Contamination-Free Workplace

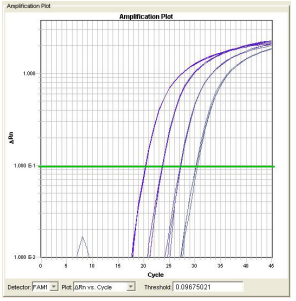
At some point, most qPCR users will experience some level of template contamination. Simple steps can mitigate the risk of accidental contamination that results in non-informative data and, thus, wasted time and money:

- Design a unidirectional process flow. PCR setup should be done in a template-free room using reagents that never come into contact with potential contamination sources. This means keeping enzyme mixes, water, primers, probes, pipettes, tubes, filter tips, and plates in a room where template is never isolated or stored.
- When mixes have been made and dispensed into the wells, move the plates to a new location for template addition.
- If using robotics, do not use the same platform for setting up PCR assay plates and isolating nucleic acids.
- Regular decontamination of commonly used equipment is recommended, especially pipettes and work surfaces.
- Regular cleaning of non-porous surfaces with a 10% bleach solution is encouraged.
- Bench-top hoods with HEPA filters and UV lights can be useful but are not absolutely necessary. Short-term UV light treatment is only effective against live organisms and not against purified nucleic acid.
- Water carboys are not recommended for long-term water storage because microorganisms can thrive in these containers.
- Multiple freeze–thaw cycles of oligonucleotides in a buffered solution are often mistakenly thought to lead to their degradation. In order to prevent this problem, users are often advised to make small aliquots of the primer-probe mixes. However, IDT has shown that PrimeTime® Assays containing primer-probe mixes are stable in buffered solutions for over 30 freeze–thaw cycles (Figure 8). Aliquots are useful if the stock solutions will be accessed frequently with pipettes that are potentially contaminated with nucleic acids.

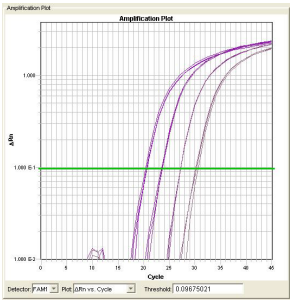
0 Freeze-Thaws



15 Freeze-Thaws

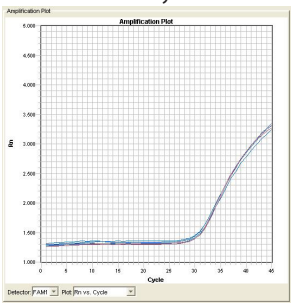


30 Freeze-Thaws



Panel A. Amplification Curves for PrimeTime® qPCR Assay.

0 vs. 30 Freeze-Thaws
Rn vs. Cycle



Panel B. PrimeTime qPCR Assay Normalized View vs. Cycle.

Figure 8. PrimeTime® qPCR Assays are Stable After 30 Freeze-Thaw Cycles. (A) A standard scale PrimeTime qPCR Assay was hydrated in IDTE to 40X. The tube was frozen (–20°C) and thawed 30 times. At 0, 15, and 30 freeze–thaws, an aliquot of the assay was run against a validated universal human reference cDNA standard curve (0.005–50 ng) using TaqMan® Gene Expression Master Mix (Applied Biosystems). (B) The PrimeTime qPCR Assays at 0.5 ng cDNA concentration in a ROX™-normalized view (Rn). PrimeTime qPCR Assays showed no probe degradation and no impact on C_q value up to 30 freeze–thaw cycles.

3. Perform Reverse Transcription

Transcription is the synthesis of RNA from a DNA template; reverse transcription (RT) is the synthesis of DNA from an RNA template. DNA synthesized from RNA is often referred to as first-strand cDNA. The conversion from RNA to cDNA is necessary because PCR uses DNA-dependent polymerases. The exact reaction conditions are dependent upon the particular kit or protocol used, but all contain the same basic components: the RNA to be converted, dNTPs to provide the nucleotides for cDNA synthesis, primers, buffer, DTT to stabilize the enzymes, RNase inhibitor to prevent RNA degradation, and a reverse transcriptase enzyme.

3.1 Sample

For accurate comparison in the qPCR step, equal amounts of starting RNA should be used from each sample in the RT reaction. Large variations in the amount of RNA between RT reactions can lead to fluctuations in RT efficiency. Poor RT may lead to loss of signal or failure to detect transcripts with low levels of expression.

3.2 Choice of Primers

The type of primers used will depend on the experimental goal. Both random primers and oligo(dT) primers will produce random cDNA, while gene-specific primers will produce cDNA for a specific target.

Random hexamer and nonamer primers bind to RNA at a variety of complementary sites and lead to short, partial-length cDNAs. These primers can be used when the template has extensive secondary structure. Random primers will produce the greatest yield, but the majority of the cDNA will be copies of ribosomal RNA, unless it is depleted prior to RT-PCR. The main advantage to using random primers is the preservation of the transcriptome so that any remaining cDNA can be used in other qPCR assays. The disadvantage is that low abundance messages may be under-represented due to consumption of reagents during cDNA synthesis of the more prevalent RNAs. Random hexamers produce a greater amount of cDNA, while random nonamers produce longer products.

Gene-specific oligonucleotide primers, which selectively prime the mRNA of interest, yield the least complex cDNA mixture and avoid reagent depletion. The main disadvantage to their use is that the cDNA produced cannot be used for assaying other genes.

Oligo(dT) primers will ensure that mRNA containing poly(A) tails are reverse transcribed. These primers are more commonly used when trying to limit the amount of ribosomal RNA being copied, or when the qPCR assays are designed to target the 3′ end of the RNA. If the mRNA is long, the 5′ end of the message may be under-represented.

3.3 Replicates and Controls

Variation can be easily introduced at this step in the process, so it is very important that all samples are treated the same including the input amount of RNA, the priming strategy, the enzyme type, the volume of the reaction, the temperature used, and the reaction time [1]. For accurate analysis of RT-qPCR results, each experiment needs to be set up with multiple replicates and controls (See Section 4.2.6, Figure 14 for a schematic outlining assay setup).

Replicates: For each experimental and control sample to be compared, it is highly recommended that at least three biological replicates are used. The number of technical replicates performed is dependent on the steps taken to minimize errors due to poor pipetting or uncalibrated equipment, and on the precision required.

No RT Control: For every reverse transcription reaction, it is important to incorporate a "no RT control" to identify erroneous signal due to genomic DNA contamination. This reaction has all of the components of the other reactions, but the reverse transcriptase is left out. This control will be very useful later in the qPCR step as a negative control.

3.4 cDNA Storage

Aliquot the cDNA samples and store the first strand cDNA at –20°C.

3.5 One-Step vs. Two-Step RT-qPCR

There are commercially available products for performing the RT reaction and qPCR in a single step (one-step qPCR). This may be a good option if you plan to use the cDNA for only a limited number of assays. However, if you are interested in making a large amount of cDNA to use for multiple assays, two-step qPCR is recommended. In addition, one-step qPCR can be less sensitive than two-step and prevents you from varying the amount of input cDNA. For more information on this subject, see the article, *Starting with RNA—One-Step or Two-Step RT-qPCR*, in the IDT *DECODED* 1.3 , October 2011 newsletter at www.idtdna.com.

3.6 Example Reverse Transcription Reaction Protocol

(20 µL reaction volume; for two-step RT-qPCR)

1. Combine the following components:

- 1 µL 2 µM gene-specific RT primer or 250 ng oligo(dT)
- 1 µL dNTP mix (10 mM each)
- 10.5 µL total RNA (10 ng/µL, 100 ng)

2. Heat at 65°C for 5 min and then chill on ice.

3. Add:

- 4 µL 5X First Strand Buffer
- 2 µL 0.1 M DTT
- 1 µL RNase inhibitor such as RNasin® Ribonuclease Inhibitor (Promega)

4. Incubate at 42°C for 2 min.

5. Add 0.5 µL Superscript® II (Life Technologies).

6. Incubate at 42–44°C for 1 hr*.

7. Incubate at 70°C for 15 min.

* For random hexamers, incubate at 25°C for 15 min to allow some extension, and then increase temperature to 42–44°C .

4. Real-Time qPCR Design and Protocols

To achieve reliable, interpretable results from qPCR the following important factors must be considered:

- Primer and probe design are crucial to the success of the experiment.
- The real-time PCR instrument will dictate certain parameters of the experiment; importantly, some instruments are not compatible with some fluorescent dyes. Table 2 (see page 35) lists several dyes and associated instrument compatibility. However, this list is limited and may be subject to change. Check the manual for your particular instrument to verify compatible dyes and correct cycling conditions.
- If you are running a multiplex experiment, additional considerations will need to be incorporated—particularly in the assay design and choice of dyes.
- As a final step before the reaction is set up, determine the controls that you will run and be sure to calculate those extra reactions into your total number of reactions. Include both positive and negative controls.

This section includes recommendations for design as well as protocols for resuspension and reaction setup. Always use RNase- and DNase-free reagents, check their expiration dates, and verify their concentrations. Although many of these considerations can be used for any 5' nuclease assay product, the protocol in Section 4.3 is intended for use with PrimeTime qPCR Assays from IDT.

4.1 PrimeTime qPCR Assays and Associated Products

PrimeTime qPCR Assays consist of 2 primers and a hydrolysis probe delivered in a single tube. These assays simplify qPCR optimization by allowing for selection of dyes, addition of a second quencher (see Section 4.1.3), choice of primer-to-probe ratios, and adjustment of qPCR design parameters. PrimeTime qPCR Assays selected from the IDT Predesigned Assay collection or generated with the RealTime PCR Design Tool for Custom Assays are guaranteed to provide assay efficiency >90% when used with a commercially available master mix and measured over a minimum of 4 orders of magnitude.

PrimeTime Custom and Predesigned qPCR Assays are offered in three different sizes to allow researchers flexibility for a range of experimental needs—from screening many genes to testing the same endogenous controls over many experiments.

4.1.1 PrimeTime Custom qPCR Assays

PrimeTime qPCR Assays can be custom designed using the sophisticated, free IDT qPCR primer and probe RealTime PCR Design Tool found in the SciTools section of the IDT website (www.idtdna.com). Simply enter the RefSeq numbers or paste in a sequence for design. The custom design tool also allows you to target specific exon locations, and will allow you to view primer and probe sequences prior to purchase. Alternatively, if you are working with human, mouse, or rat genes, IDT has predesigned assays covering those transcriptomes that can be located using a simple, searchable format (see Section 4.1.2 below).

4.1.2 PrimeTime Predesigned qPCR Assays

IDT offers PrimeTime Predesigned qPCR Assays for human, rat, and mouse targets. The IDT design engine incorporates numerous parameters optimized to yield a robust qPCR assay. The design process uses up-to-date sequence information from the NCBI RefSeq database and accurate Tm and secondary structure prediction to protect against off-target amplification and avoid SNPs. SNPs that occur in 1% or more of the human population are present in the genome on average every few hundred bases [16]. Keeping up to date with the continually increasing number of annotated SNPs is critical for qPCR experimental success. The frequently updated design engine used to generate PrimeTime Predesigned qPCR Assays allows researchers to focus on the experiment rather than the design.

4.1.3 ZEN Double-Quenched Probes

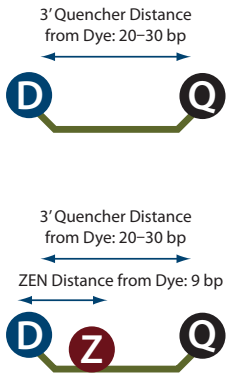


Figure 9. Location of ZEN™ Quencher.

IDT has developed an internal ZEN quencher for the production of double-quenched probes that produce qPCR data with less background and increased signal. Double-quenched probes contain a 5' fluorophore choice of FAM, HEX, JOE, TET, or MAX; a 3' IBFQ quencher; and an internal ZEN quencher. While the distance between fluorophore and quencher in traditional probes is 20–30 bases, the internal ZEN quencher decreases this distance to only 9 bases (Figure 9). This shortened distance, particularly when combined with the traditional 3' end quencher, leads to extremely efficient quenching with significantly less background (Figure 10), and enables the use of much longer probes for designs falling within AT-rich target re-

gions. In addition to the greatly decreased background, the double-quenched probes allow users to experience increased sensitivity and greater precision in their qPCR experiments. Double-quenched probes are available as PrimeTime Custom qPCR Assays, PrimeTime Predesigned qPCR Assays, or sequences specified by the customer.

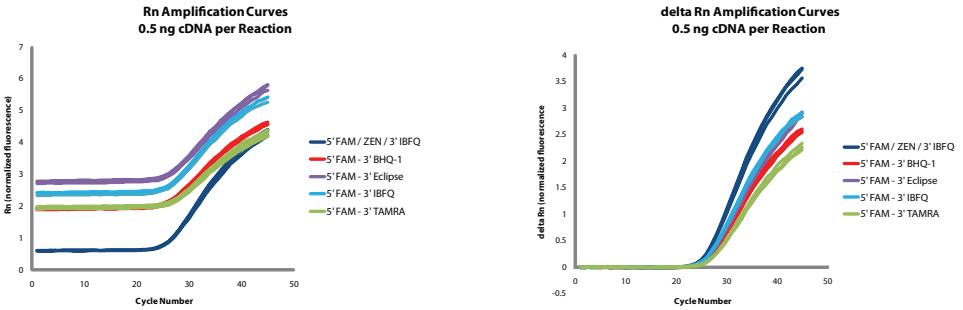


Figure 10. ZEN™ Dual-Quenched Probes Provide Superior Sensitivity. 5'FAM probes with five different quenchers: ZEN/ Iowa Black® FQ (ZEN/IBFQ), Black Hole Quencher® (BHQ), Eclipse®, Iowa Black FQ (IBFQ), and TAMRA were synthesized to target the ACTB locus. qPCR was carried out in triplicate using each probe, a primer pair for ACTB, and 0.5ng of cDNA. All reactions were performed using Applied Biosystems TaqMan® Gene Expression Master Mix under standard cycling conditions on the Applied Biosystems 7900HT Real-Time PCR instrument.

Related Products at IDT

SciTools® Web Tools: There are several free design and analysis tools available on the IDT website.

- PrimeTime® Predesigned qPCR Assay Selection Tool—Use to select assays for human, mouse, and rat targets
- RealTime PCR Design Tool—Use to design primers, probes, and assays for gene targets in other species
- OligoAnalyzer® Tool—Use to analyze oligonucleotide melting temperature, hairpins, dimers, and mismatches
- UNAFold Tool—Use to analyze oligonucleotide secondary structure

For more information, and to use these free SciTools Web Tools, see Section 8.2.1 and the SciTools webinar under the Support tab on the IDT website (www.idtdna.com).

4.2 5′ Nuclease Assay Design

4.2.1 General Design Considerations

Know your gene: A well-designed assay begins with an understanding of the gene of interest, including knowledge of the transcript variants and their exon organization. Use databases such as Ensembl or GenBank to identify exon junctions, splice variants, and locations of single-nucleotide polymorphisms (SNPs). For genes that have multiple transcript variants, align related transcripts to understand exon overlap using a program such as Clustal, or use NCBI online tools such as the Gene Viewer. For transcript-specific designs, target primers and probes within exons unique to the transcripts of interest, and BLAST primer and probe sequences to ensure they do not occur in related transcripts or cross-react with other genes within the species (see below). For splice-common designs, target primers and probes within exons found across all transcript variants. Note that the PrimeTime Predesigned qPCR Assay Design Tool takes these factors into consideration for you (see Section 8.2.1a).

Avoid SNPs: With the increased focus on high-throughput sequencing, the number of identified SNPs in the human genome is rapidly increasing. In the human genome, SNPs are present in at least 1% of the population and occur on average once every few hundred bases. A single mismatch between primer and target, due to a SNP, can significantly decrease the melting temperature of that hybrid (by up to 10°C), affecting the efficiency of the PCR, and ultimately the interpretation of experimental results.

Ensure Specificity: Ensure that both the primers and the probe are specific to the target and not complementary to other sequences. Use BLAST to analyze the sequences to ensure their specificity. BLAST is a Basic Local Alignment Search Tool provided by NCBI (http://www.ncbi.nlm.nih.gov/) that finds regions of local similarity between sequences. Enter an accession number or the sequence in FASTA format and compare it to the genome of a specific species or to all BLAST databases. BLAST allows searches against nucleotide or protein databases and provides the statistical significance of the matches (for more information, see the article *Tips for Using BLAST to Locate PCR Primers* in the IDT *DECODED* 1.1, April 2011 newsletter).

Working with Limited Samples or Low Abundance Targets: For two-step RT-qPCR protocols, the input amount of cDNA used for qPCR can be regulated to increase the

amount of target available for detection. This is useful when working with low abundance targets when sample is not limited.

When sample is limited, preamplification of RNA (by linear, isothermal amplification), or first strand cDNA prior to qPCR, can increase the amount of detectable target for low abundance transcripts from minute amounts of sample. This extra step is often incorporated when performing single cell analysis or working with clinical samples, fine needle biopsies, laser captured microdissection samples, or FACS generated cells. IDT can supply custom pooled gene-specific primer mixes for this application.

4.2.2 Primers and Probes

Use the PrimeTime Predesigned Assay Selection Tool to identify human, mouse, or rat assays. We strongly recommend using the RealTime PCR Design Tool located on the IDT website for custom assays (i.e., assays for genes of other species) to be sure all of the important parameters are included in assay design. IDT recommendations for primers, probe, and amplicon specifications are provided in the following text and summarized in Table 1.

	Primers		*Probe*		*Amplicon*	
	Range	*Ideal*	*Range*	*Ideal*	*Range*	*Ideal*
Length	18-30	22	20-28*	24	70-150	100
Melting Temperature	60-64°C	62°C	66-70°C	68°C	NA	NA
GC content	35-65%	50%	35-65%	50%	NA	NA

*For probes that do not contain MGB T_m enhanced properties.

Table 1. Recommended Specifications for Primers, Probes, and Amplicons. These specifications are based on a final reaction composition of 50 mM KCl, 3 mM $MgCl_2$, and 0.8 mM dNTPs.

4.2.2a Primers

T_m: Typically, an annealing temperature of 60°C is used during PCR. The optimal melting temperature of the primers is between 60 and 64°C, with an ideal temperature of 62°C, which is based on the average conditions and factors associated with the PCR. The melting temperature of the two primers should not differ by more than 4°C in order for both primers to bind simultaneously and efficiently amplify the product.

Length: Aim for primer lengths of 18–30 bases with a balance between the melting temperature, purity, specificity, and secondary structure considerations.

GC content: Ensure that the primers are specific to the target and that they do not contain regions of four or more consecutive Gs [17]. The GC content should be within the range of 35–65%, with an ideal content of 50%, which allows complexity while still maintaining a unique sequence. Avoid sequences that may create secondary structures, self-dimers, and heterodimers; use programs such as the IDT OligoAnalyzer tool to find potential sites that are likely to form these structures.

4.2.2b Probes

T_m: The melting temperature of the probe should be 6–8°C higher than the primers and should fall within the range of 66–70°C for a standard two-step protocol. If the melting temperature is too low, the probe will not bind to the target. In this case, the primers may amplify a product, but as the probe is not bound to a target, it will not be proportionally degraded and, thus, will be unable to provide the fluorescence that is necessary to detect the product.

Length: The length of a single-quenched probe should be 20–30 bases to achieve an ideal T_m without increasing the distance between the dye and quencher such that the quencher will no longer absorb the fluorescence of the dye (probes with a single terminal quencher that are longer than 30 bases may perform poorly due to the distance between the quencher and dye). Double-quenched probes that include the IDT ZEN molecule as a secondary, internal quencher allow longer probes to be used while providing strong quenching and increased signal. See Section 4.1.3 for more information.

GC content: Aim for a GC content of 35–65% and avoid a G at the 5′ end to prevent quenching of the 5′ fluorophore. As with the primers, avoid sequences that may create secondary structures or dimers.

Location: Ideally, the probe should be in close proximity to the forward or reverse primer, but not overlap, although this is not absolutely necessary. Probes can be designed to bind to either strand of the target.

4.2.2c Amplicons

Length: Design amplicons of 70–150 bp, which will allow the primers and probe to compete for hybridization and provide a sequence that is long enough for all components to bind. This length is most easily amplified using standard cycling conditions. Longer amplicons of up to 500 bases can be generated, but cycling conditions will need to be altered to account for the increased extension time. Amplicons or assay designs should span an exon–exon junction to reduce the possibility of genomic contamination.

T_m: Calculate all melting temperatures under real-time PCR conditions—standard parameters for qPCR are 50 mM K^+, 3 mM Mg^{2+}, and 0.8 mM dNTPs: however they can vary widely from this, particularly with respect to Mg^{2+} concentration. See Section 4.2.2d, below, for more information on calculating melting temperature.

4.2.2d Calculating Melting Temperature (T_m)

During the annealing step of PCR, primers and probes hybridize to targets, forming short duplexes. The stability of these duplexes is described by the melting temperature, the temperature at which an oligonucleotide duplex is 50% single-stranded and 50% double-stranded (Figure 11).

Melting temperature is a key design parameter. Inaccurate T_m predictions increase the probability of failed assay design. IDT provides several free software tools (SciTools applications) on the website that can predict T_m values from oligonucleotide sequences and reaction composition. It is often mistakenly believed that T_m is solely a property of the oligonucleotide sequence and independent of experimental conditions. Melting temperature depends on oligonucleotide sequence, oligonucleotide concentration, and cations present in the buffer, specifically monovalent ($[Na^+]$) and divalent ($[Mg^{2+}]$) salt concentrations (Figure 12). For this reason, the melting temperature for specific experimental conditions should be calculated using IDT SciTools design tools (see Section 8.2.1 for information on which SciTools application best fits your needs).

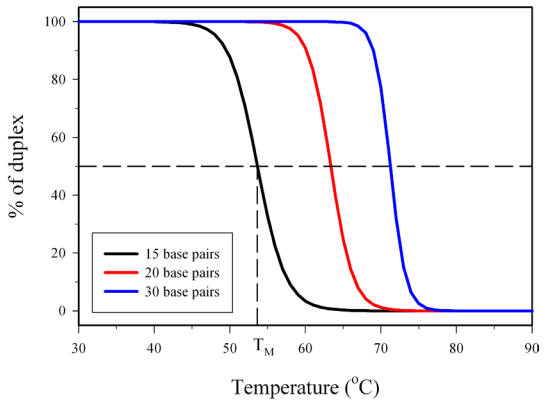


Figure 11. Melting Profiles for Primers of Various Lengths. Reactions were performed using a PCR buffer containing 1 mM Mg^{2+}, 50 mM KCl, 10 mM Tris. GC content for all primers was ~50%.

Predictive algorithms have recently been significantly improved; the nearest-neighbor method predicts T_m with a higher degree of accuracy than previously used methods [18]. Older formulas, which do not take into account interactions between neighboring base pairs, provide T_m predictions that are too inaccurate for real-time PCR design. IDT scientists have published experimental studies on the effects of Na^+, K^+ and Mg^{2+} on the stability of oligonucleotide duplexes and have proposed a model with greater accuracy [19]. The linear T_m correction has previously been used to account for salt stabilizing effects, but melting data from a large oligonucleotide set demonstrated that non-linear effects are substantial and must be considered [19].

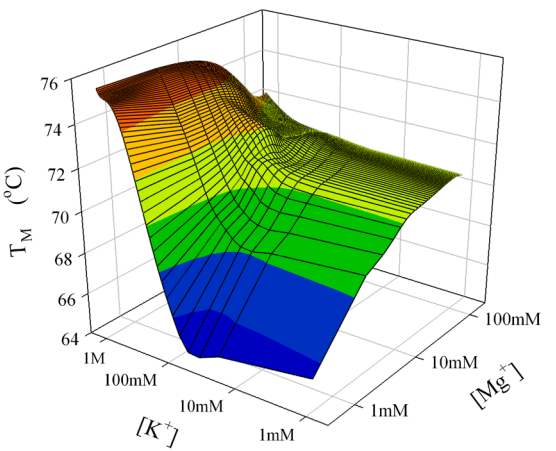


Figure 12. Salt Concentration Affects T_m. The stability of a 25 bp duplex (CTG GTC TGG ATC TGA GAA CTT CAG G) varies with K^+ and Mg^{2+} concentrations. Competitive binding of ions to DNA is observed.

PCR buffers also contain deoxynucleoside triphosphates (dNTPs), which bind magnesium ions (Mg^{2+}) with much higher affinity than DNA. Since they decrease the activity of free Mg^{2+}, T_m may be also decreased [19] (Figure 12). The best predictive algorithm considers this effect as well. IDT SciTools design tools (see below) employ the latest nearest-neighbor method, thermodynamic parameters [20–22] and the improved salt effects model [19] to achieve state-of-the-art predictions of melting temperatures with an average error of approximately 1.5°C.

Related Products at IDT

SciTools® Design Tools: A number of free design and analysis tools are available on the IDT website. These include the RealTime PCR Design Tool (for designing primers, probes, and assays), OligoAnalyzer® Tool (for analyzing oligonucleotide melting temperature, hairpins, dimers, and mismatches), and mFold (for analyzing the secondary structures of oligonucleotides). For more information and to use these free SciTools programs, visit www.idtdna.com.

4.2.3 Choosing the Correct Reporter Dye for the Instrument

The choice of fluorescent dye will depend on the instrument you are using and the compatibility of the dye with the instrument. Table 2 lists dyes recommended by IDT that are compatible with common real-time PCR instruments. Refer to your instrument manufacturer's guidelines for information specific to your particular instrument. FAM is the most popular of these dyes and is often more sensitive than some of the other dyes available.

Instrument Compatibility with Reporter Dyes

	FAM	TET	HEX	JOE	MAX	Cy3	TYE 563	TAMRA	ROX	LC Red 610	Texas Red	TEX 615	LC 640	Cy5	TYE 665
ABI 7000	•	o	o	•	o	x	x	•	x	x	x	x	x	x	x
ABI 7300	•	o	o	•	o	x	x	•	x	x	x	x	x	x	x
ABI 7500	•	o	o	•	o	•	o	•	•	o	•	o	o	•	o
ABI 7900	•	•	o	•	o	x	x	x	x	x	x	x	x	x	x
ABI StepOne	•	o	o	•	o	x	x	x	x	x	x	x	x	x	x
ABI StepOne Plus	•	o	o	•	o	x	x	•	x	x	x	x	x	x	x
BioRad CFX384	•	o	•	o	o	o	o	o	o	o	•	o	o	•	o
BioRad CFX96	•	o	•	o	o	o	o	o	o	o	•	o	o	•	o
BioRad iCycler	•	o	•	o	o	o	o	o	o	o	•	o	o	•	o
BioRad MiniOpticon	•	o	•	o	o	x	x	x	x	x	x	x	x	x	x
BioRad MyIQ2	•	o	•	o	o	x	x	x	x	x	x	x	x	x	x
BioRad MyIQ5	•	o	•	o	o	o	o	•	o	o	•	o	o	•	o
Cepheid Smartcycler	•	•	o	o	o	•	o	x	o	o	•	o	o	•	o
Eppendorf Mastercycler	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Qiagen Rotorgene Q	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Roche LC480	•	o	•	o	o	o	o	o	o	•	o	o	•	•	o
Roche LC1536	•	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Roche LC Nano	·	o	·	o	o	o	o	o	o	·	·	o	o	·	o
Stratagene Mx3000P	•	o	•	o	o	•	o	o	o	o	•	o	o	o	o
Stratagene Mx3005P	•	o	•	o	o	•	o	o	o	o	•	o	o	•	o

Table 2. Instrument Compatibility with Reporter Dyes.

•	*supplier provided or recommended reporter dyes*
o	*instrument capable dyes, but may require calibration*
x	*instrument incapable of supporting*

4.2.4 Multiplex qPCR

In multiplex PCR, multiple targets are amplified in a single reaction tube. Each target is amplified by a different set of primers and a uniquely-labeled probe that will distinguish each PCR amplicon. Multiplexing provides some advantages over single-reaction PCR, including requiring a lower amount of starting material, increased throughput, lowered reagent costs, and less sample handling. However, the experimental design for multiplexing is more complicated because the amplification of each target can affect others in the same reaction. Therefore, careful consideration of design and optimization of the reactions is critical. In order to incorporate all necessary parameters, we strongly recommend using a design tool for primers and probes. Suitable tools can be found in the SciTools applications section of the IDT website. See the Section 8.2.1 for more information.

1. Ensure that the primers and probe sets are not complementary to each other. Use BLAST to analyze the sequences and ensure they are non-complementary. For help with using the BLAST tool, see *Tips for Using BLAST to Locate PCR Primers*, in the IDT DECODED 1.1, April 2011 newsletter.

2. Each target must be identified by a separate reporter dye. Select dyes with little or no overlap in their emission spectra (Table 3). Some instruments are compatible with only certain dyes—read Section 4.2.3, so check the documentation for your instrument to ensure the dyes are compatible. As a general rule, it is a good idea to select FAM for any low copy transcripts because it has a strong signal. Lower signal fluorophores can then be used for the more abundant transcripts.

3. Minimize signal cross-talk by using high quenching probes such as the IDT ZEN double-quenched probes.

4. Optimize the individual reactions and ensure that they each have an efficiency >90%.

5. Validate the multiplex reactions by running a combined reaction alongside an individual reaction to ensure similar performance. Compare the standard curves and verify that the C_q values are similar at both the high and low ends. A good multiplex should have similar curves and similar limits of detection (Figure 13).

6. Optimize the multiplex reactions. Limit the primers for targets expressed at a high level to a 1:1 primer-to-probe ratio. Increase the primer-to-probe ratio for targets expressed at lower levels. IDT offers custom primer-to-probe ratio options for PrimeTime qPCR Assays. Increasing the amount of enzyme and dNTPs added to

the reaction may be necessary—we recommend doubling the amount of these reagents. There are a few commercially available master mixes that are specially formulated for multiplexing.

Dye	Dye Absorption max (nm)	Dye Emission max (nm)
6-FAM	495	520
TET	522	539
HEX	538	555
JOE	529	555
MAX	524	557
Cy3	550	564
TAMRA	559	583
ROX	588	608
TEXAS RED	598	617
TEX 615	596	613
TYE 665	645	665
Cy5	648	668

Table 3. Absorbance Range of Fluorophores.

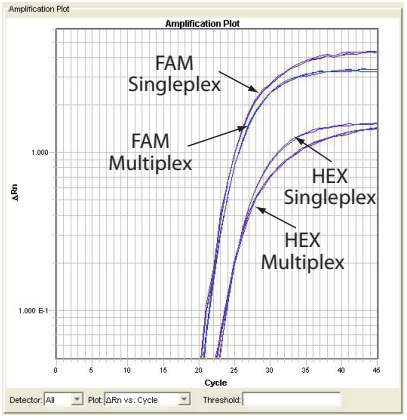


Figure 13. Multiplex PrimeTime qPCR Assays. Each reaction was performed using 50 ng cDNA with FAM or HEX dye. C_q values were 22.8 for FAM singleplex, 22.5 for FAM multiplex, and 25 for HEX singleplex and multiplex.

4.2.5 Calibrating Dyes for Multiplex qPCR

Multiplex qPCR allows researchers to perform multiple assays simultaneously in a single well. The advantages of this technique include increased efficiency in sample and resource usage, higher throughput and faster time to results, and elimination of well-to-well variations. In multiplex qPCR, fluorescent dyes with distinct spectra are used to detect individual targets. Thermal cycler and real-time detection system emission and excitation filter settings vary from manufacturer to manufacturer, so the instrument must be calibrated for each dye as part of the experiment optimization process to enhance dye specificity and minimize background and overlapping of fluorescent signals.

General Calibration Protocol

Calibration procedures are specific to each real-time PCR instrument. In general, most calibration protocols require the following steps:

- Perform background calibration procedure
- Dilute dye in amount of reaction buffer recommended by the manufacturer
- Aliquot amount of recommended dilution into a PCR plate and seal (use different plates for each dye to be calibrated)
- Follow instructions in the instrument software menu to perform calibration for each dye
- Review data to identify variations in dye spectra

Refer to the instrument manufacturer for specific instructions.

4.2.6 Replicates and Controls

For accurate analysis of qPCR results, each experiment needs to be set up with multiple replicates and controls (Figure 14).

4.2.6a Replicates

qPCR experiments use two different types of replicates. Technical replicates (repeated RT or qPCR reactions) are used to compensate for technical noise and to increase the precision of the qPCR results. These repeated measures should not be used for statistical

testing of biological hypotheses. Biological replicates (repeated cell cultures, or different individuals or specimens) are required to draw biologically relevant conclusions from your experiments.

The number of replicates depends on the specific needs of the experiment. For each experimental and control sample to be compared, at least 3 biological and 3 technical replicates are necessary to minimize errors in measured gene expression due to pipetting. And at least 3 biological replicates are required if you want to draw statistical conclusions. See Figure 14 for an example.

However, if you are interested in small expression differences or require higher confidence (e.g., for diagnostics), it is useful to include more replicates. Most studies require larger numbers of samples. On the other hand, when performed by experienced users, large screening studies that include many samples, and fewer replicates, may suffice.

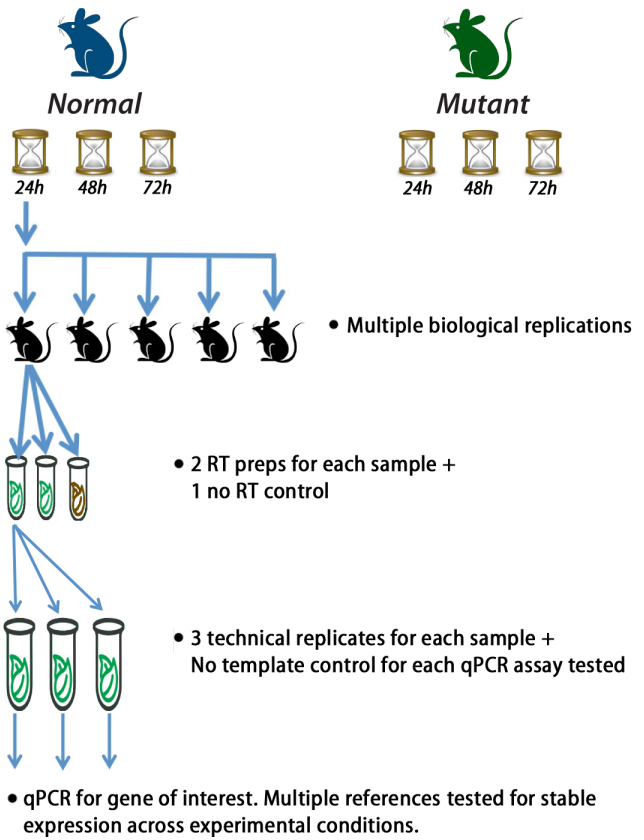


Figure 14: qPCR Assay Setup. Outline of the replicates and controls needed for an experiment comprising two different samples examined at several time points after treatment. Include 3–5 biological replicates for each time point studied. For each biological replicate studied, perform 2 reverse transcription reactions (+RT) and 1 with no RT (–RT control). For each cDNA sample generated, set up 3 technical replicates for qPCR analysis. Include a No Template Control for each gene analyzed to identify any signal due to contamination.

4.2.6b Negative Controls

IDT recommends the following three negative controls. The no template control is an absolute requirement for all qPCR experiments.

1. A no template control (NTC) omits DNA from the PCR. This reaction serves as a general control for unwanted nucleic acid contamination or primer-dimer formation that may make the results more difficult to interpret, particularly when using SYBR® Green I dye. Perform this control for each assay.
2. A no reverse transcriptase control (–RT) omits the reverse transcriptase in the reverse transcription step of a qRT-PCR. The purpose of this control is to assess the amount of genomic DNA contamination present in an RNA preparation. Perform this control for any assay that might amplify genomic DNA.
3. A no amplification control omits the DNA polymerase from the PCR. This reaction serves as a control for background fluorescence of the PCR assay and probe stability.

4.2.6c Positive Controls

1. An exogenous positive control is external DNA or RNA carrying a target of interest. These control reactions will alert you to any components in the sample that might inhibit reverse transcription and/or PCR. IDT synthesizes gBlocks™ Gene Fragments, MiniGene™ Synthetic Genes, and Ultramer™ Oligonucleotides, which can serve as exogenous positive controls with known starting copy number [23].
2. An endogenous positive control is a native target that is present in the experimental sample of interest and can serve as a normalizer among samples. These control reactions will correct for quantity and quality differences between samples. IDT recommends that you test at least two, but preferably three, normalizing or reference genes to ensure accurate internal controls. The most appropriate normalizing gene to use will depend on the RNA source and experimental conditions of the sample you will be testing. It is best practice to screen multiple genes under the experimental conditions employed to determine which expression levels fluctuate the least. Programs, such as geNorm, can be used to evaluate the performance of various normalizers. The most commonly used normalizers are listed in Table 4 [24]. Alternatively, review the literature for the genes tested on samples with conditions similar to yours. If you choose a normalizing gene from the literature, be sure to perform a reaction to verify that the gene expression levels do not fluctuate across samples before you use it as a control.

GENE ID	Description
18S	18S ribosomal RNA
ACTB	actin, beta
ALDOA	aldolase A,fructose-bisphosphate
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
B2M	beta-2-microglobulin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GUSB	glucuronidase, beta
HMBS	hydroxymethylbilane synthase
HPRT1	hypoxanthine phosphoribosyltransferase 1
HSPCB	heat shock 90kDa protein 1, beta
IPO8	importin 8
LDHA	lactate dehydrogenase A
NONO	non-POU domain containing, octamer-binding
PGK1	phosphoglycerate kinase 1
PGK1	phosphoglycerate kinase 1
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A
PPIA	peptidylprolyl isomerase A (cyclophilin A)
RPL11	ribosomal protein L11
RPL19	ribosomal protein L19
RPL32	ribosomal protein L32
RPLP0	ribosomal protein, large, P0
RPS18	ribosomal protein S18
RPS27A	ribosomal protein S27a
SFRS9	splicing factor, arginine/serine-rich 9
TBP	TATA box binding protein
TFRC	transferrin receptor
UBC	ubiquitin C
YWHAZ	3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Table 4. Genes Commonly Used for Normalization.

Related Products at IDT

Both Ultramer™ Oligonucleotides and gBlocks™ Gene Fragments serve as excellent controls and, importantly, can serve as standards of known concentration. For more information and to order Ultramer Oligonucleotides and gBlocks Gene Fragments visit the IDT website at www.idtdna.com.

Ultramer™ Oligonucleotides: IDT synthesis systems and chemistries allow high-fidelity synthesis of very long oligonucleotides (up to 200 bases). Ultramer Oligonucleotides are suitable for demanding applications such as cloning, shRNA, mutagenesis, and gene construction, greatly reducing effort and time by having entire target fragments synthesized by IDT.

gBlocks™ Gene Fragments: IDT offers double stranded DNA fragments up to 500 bp in size. gBlocks fragments are constructed using Ultramer Oligonucleotides and are sequence-verified.

4.3. PrimeTime® qPCR Assay Protocol

This protocol is intended for use with IDT PrimeTime qPCR Assays. Concentrations and volumes may vary for other products.

4.3.1 Resuspension Protocol

1. Centrifuge PrimeTime qPCR Assay tubes at 750 x g for 10 sec prior to opening in case any material was dislodged during shipment.
2. Resuspend the assay in IDTE buffer (10 mM Tris, 0.1mM EDTA, pH 8.0) at the volumes indicated in Table 5.
    a. Resuspend PrimeTime qPCR Assays as 40X, 20X, or 10X stocks. A particular concentration may be preferred depending on the size of the assay and desired final reaction volume (see Table 5 below).
    b. Vortex the sample to ensure maximal product recovery.
3. Centrifuge the resuspended assay at 750 x g for 10 sec. The resuspended assays will yield a final 1X concentration of 500 nM primers and 250 nM probe when ordered using default conditions.*

* Standard or XL Assays ordered using a custom primer-to-probe ratio other than 2:1 will yield a different concentration of primers.

Recommended Resuspension Volumes for PrimeTime Assay Stock Creation

	Final Desired Stock Concentration		
	40X	*20X*	*10X*
PrimeTime Mini qPCR Assay	Not recommended	100 µL	200 µL
PrimeTime Std qPCR Assay	250 µL	500 µL	1,000 µL
PrimeTime XL qPCR Assay	1,250 µL	Not recommended	Not recommended

Table 5. Recommended Resuspension Volumes for Producing PrimeTime qPCR Assay Stock Solutions.

Related Products at IDT

IDTE buffer: IDT offers 1X TE Buffer (10 mM Tris, pH 7.5 or 8.0, 0.1 mM EDTA) for initial resuspension and storage of DNA oligonucleotides. DNA oligonucleotides can be damaged by prolonged incubation or storage in even mildly acidic solutions; DNA dissolved in distilled water often has a final pH <5.0 and is at risk of depurination. IDTE is guaranteed to be nuclease-free. Each lot is tested using our RNaseAlert and DNaseAlert Kits to document the absence of nuclease activity. For more information and to order IDTE buffer, visit the IDT website at www.idtdna.com.

4.3.1a Avoiding Probe Degradation

In order to avoid probe degradation, resuspend primers and probes in TE buffer (10 mM Tris; 0.1 mM EDTA; pH 8.0) rather than water as TE buffer will maintain a constant pH. Store probes away from light. Oligonucleotides should be distributed into aliquots for immediate use or long-term storage. Storage in aliquots will help minimize the risk of contamination of the stock solutions.

4.3.2 Assay Protocol

Most qPCRs are performed in 20 µL, 10 µL, or 5 µL reaction volumes. It is very important that the cDNA and reaction volumes remain constant across all samples that need to be compared. IDT recommends that you perform a minimum of triplicate reactions for each sample. Remember to include control samples when calculating the number of reactions you need for each assay.

1. In a sterile 1.5 mL microcentrifuge tube, pipette the assay, master mix, and water in the volumes listed based on the number of assays you need to run (Table 6). Cap the tube and vortex to mix. Centrifuge the tube at 750 x g for 10 sec.
2. Depending on the number and total volume of reactions, select 96- or 384-well plates.
3. Transfer the appropriate amount of master mix reaction into each well of the reaction plate.
4. Add the cDNA to the appropriate wells. Pipette up and down to mix the reaction.
5. Seal the plate with optically clear film.
6. Centrifuge the plate at 1000 x g for 30 sec. Load the plate on the instrument.
7. The suggested real-time PCR cycling times are 3–10 min 95°C; 40 x (15 sec 95°C, 45 sec 60°C). This is a typical protocol for qPCR done under standard cycling conditions. If a fast master mix is being used, it is imperative to adopt the cycling conditions recommended by the manufacturer. This will result in optimal activation of polymerase present in the master mix. The exact cycling parameters will always depend on the particular master mix and instrument being used. Refer to the instrument manufacturer's guidelines and the instructions for the master mix you are using for correct cycling conditions. Using the correct cycling conditions to activate the enzyme is crucial to the success of the experiment.

10X Assay

	20 µl reaction		10 µl reaction		5 µl reaction	
PCR reaction component	1 rxn	3 replicates*	1 rxn	3 replicates*	1 rxn	3 replicates*
10X PrimeTime Assay	2 µL	8 µL	1 µL	4 µL	0.5 µL	2 µL
2X Master Mix**	10 µL	40 µL	5 µL	20 µL	2.5 µL	10 µL
cDNA‡ + RNase-free water (IDT)‡‡	8 µL	32 µL	4 µL	16 µL	2 µL	8 µL

20X Assay

	20 µl reaction		10 µl reaction		5 µl reaction	
PCR reaction component	1 rxn	3 replicates*	1 rxn	3 replicates*	1 rxn	3 replicates*
20X PrimeTime Assay	1 µL	4 µL	0.5 µL	2.0 µL	0.25 µL	1 µL
2X Master Mix**	10 µL	40 µL	5 µL	20 µL	2.5 µL	10 µL
cDNA‡ + RNase-free water (IDT)‡‡	9 µL	36 µL	4.5 µL	18 µL	2.25 µL	9 µL

40X Assay

	20 µl reaction		10 µl reaction		5 µl reaction	
PCR reaction component	1 rxn	3 replicates*	1 rxn	3 replicates*	1 rxn	3 replicates*
40X PrimeTime Assay	0.5 µL	2 µL	0.25 µL	1 µL	0.13 µL	0.5 µL
2X Master Mix**	10 µL	40 µL	5 µL	20 µL	2.5 µL	10 µL
cDNA‡ + RNase-free water (IDT)‡‡	9.5 µL	38 µL	4.75 µL	19 µL	2.38 µL	9.5 µL

* Replicates include excess to account for volume loss during pipetting.
‡ IDT recommends 1-100 ng of cDNA per 20 µL reaction.
‡‡DEPC-free, nuclease-free IDT water is recommended.
** It is imperative that every single sample be treated in the same manner in order to achieve reliable results. For that reason, it is very important to use a mix to ensure that every sample receives the same amount of each of the reaction components. See Figure 15 for information on all master mixes tested at IDT.

Table 6. Master Mix Volumes for Number of Assays Planned.

4.3.2a Master Mixes

Master mixes prepared by manual addition of components (reaction buffer, dNTPs, $MgCl_2$, and Taq polymerase) allow maximum flexibility because components can be adjusted according to experimental needs. However, most researchers use commercial master mixes optimized to work in most standard assays. It is important to note that small changes in the master mix formulation can significantly affect assays, and some formulations are designed for use with specific cycling conditions (commercial fast master mixes should always be used with the specified fast cycling conditions). A brief description of the role of the different components in a master mix is given below.

- Buffer—required to maintain optimum pH and salt conditions.
- $MgCl_2$—required to stabilize primer and probe interactions with DNA and as a cofactor for Taq polymerase. Occasionally, it may be necessary to add more $MgCl_2$ to the master mix in order to achieve optimum amplification results.
- dNTPs—The building blocks for DNA synthesis. Some master mixes include dUTP in conjunction with UNG enzyme to eliminate carryover of product from a previous PCR. However, dUTP is not incorporated as efficiently as dTTP and might affect amplification.
- UNG (Uracil N-glycosylase) enzyme—Used to eliminate PCR carryover contamination by degrading any PCR product with incorporated uracil bases. If a master mix includes dUTP, it is necessary to adapt cycling conditions to include an UNG step to degrade any previous PCR product.
- Polymerase—Master mixes normally contain modified DNA polymerases to eliminate nonspecific priming that may occur before the initial denaturation step when a standard DNA polymerase is used. Taq polymerases have been modified—through use of an antibody or chemical modification, or aptamers—to be inactive at low temperatures. These modified enzymes are known as "hot start" polymerases and require heating at 95°C for 2–10 minutes for activation.
- ROX—Some thermal cyclers require the use of an internal reference dye, such as ROX, for normalization across wells and to account for pipetting errors. Therefore, certain master mixes are available with different formulations of ROX. Your instrument user manual should tell you if ROX dye is required and how much is recommended in your assay.

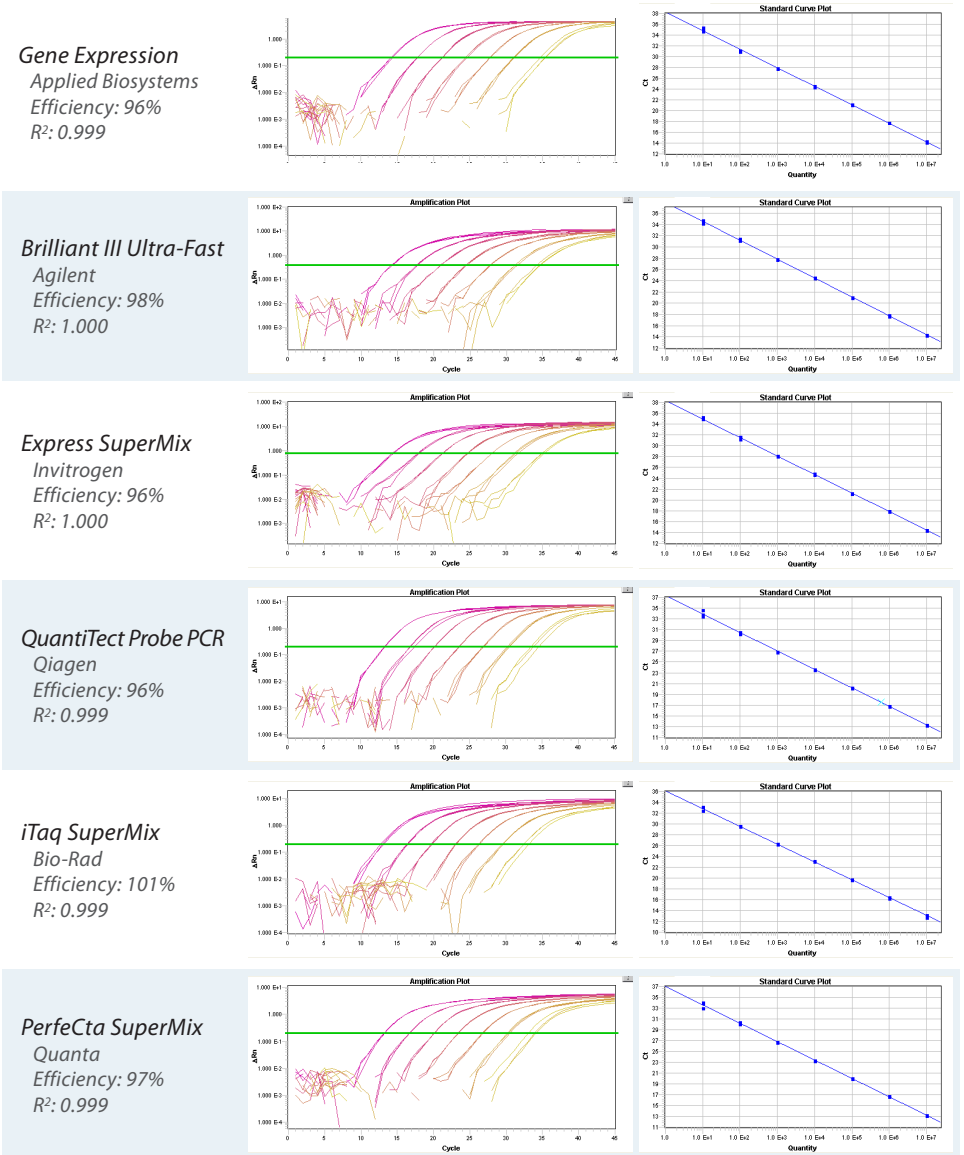


Figure 15. Successful Amplification of PrimeTime® Assays Using Various Commercial Master Mixes. IDT tested 15 different PrimeTime Predesigned qPCR Assays with 6 master mixes across 7 orders of magnitude (10X dilutions from 1 x 10^7 to 10 copies). The results for one of the 15 assays tested (ATAD2 human ATPase transcript; NM_014109) are shown. All master mixes were tested using 12 µL reactions (500 nM primers and 250 nM Double-Quenched Probe) on an Applied Biosystems 7900HT Real-Time PCR System for 45 cycles. Cycle settings and reference dye were set as recommended by each master mix manufacturer. Thresholds were set to best match the amplification of each individual assay. The data demonstrate greater than 90% efficiency and correlation coefficients >0.99 for all qPCR master mixes tested.

5. Assay Validation

Before valuable experimental samples are consumed in the qPCR analysis phase, it is important to have carefully evaluated the assay performance for specificity and efficiency. It is important to validate each new assay—even if it has previously been reported in the literature or is commercially available—to verify that the assay is working according to standards under the conditions you will be using.

5.1 Specificity Analysis

It is important to validate assay specificity to ensure that only the sequence of interest is detected. Determine specificity using one of 3 different characteristics: the melt curve, size, or sequence of a PCR product.

5.1.1 Melt Curves

When performing assays that use intercalating dyes as the detection chemistry, you can use melt curves to analyze the specificity of the assay. Melt curves are characteristic for the PCR products generated. Therefore, if multiple PCR products are formed, e.g., as a result of nonspecific amplification, multiple melt peaks will likely be present. Specific amplification is typically characterized by a unique melt peak, although unique products may show multiple peaks if the sequence contains domains that melt out at different temperatures. Predict whether this will be the case using a software package such as uMelt; available at http://dna.utah.edu/umelt/umelt.html.

5.1.2 Amplicon Size Analysis

By performing size analysis of a PCR product on agarose gels or microfluidic devices, you can detect any nonspecific products that have formed. It is possible for two different products to be of the same size, e.g., in case of co-amplification of homologous sequences (pseudogenes, members of same gene family, etc.). These events can be detected by combining melt curve and size analysis or performing the more detailed sequencing analysis.

5.1.3 Sequencing

Sequencing of PCR products, especially using massively parallel (deep) sequencing, provides the most thorough validation of specificity, but at significant cost and effort.

5.2 Efficiency Analysis

5.2.1 Standard Curves

There are 2 types of standard curves: absolute or relative standard curves. An absolute standard curve is created by diluting a nucleic acid sample (typically a plasmid, oligonucleotide, or purified PCR product) that has been accurately quantified by other means. A dilution series of this sample is prepared and each dilution serves as a standard. To obtain the most accurate quantification, the amplification efficiency of the standards must be equivalent to that of the test samples. The standards are assayed simultaneously with the test samples and a standard curve is generated from the dilutions. The concentration or number of copies of the test samples can then be determined through interpolation from the standard curve. Creating a standard curve requires setting up additional reactions; however, the data obtained can be very important for determining the quality of the PCR.

Relative standard curves are generated by serially diluting a sample whose target concentration is not known (e.g., cDNA prepared from a total RNA sample for which the concentration of the different genes is not known). Such relative standard curves are typically used for assay performance assessment and quality control of the experiment.

Creating a standard curve requires setting up additional reactions; however, the data obtained can be very important for determining the quality of the PCR. Researchers experienced in qPCR often consider it valid to assess assay performance using a standard curve only once and then never use a standard curve again, particularly in a research setting where many different assays are used.

5.2.2 Range of Dilution

A standard curve across multiple $\log_{10}$ units is needed (Figure 16). The concentrations should span a minimum of 4 $\log_{10}$ of magnitude, but preferably 5–6 $\log_{10}$. The concentrations of the test unknowns should fall within the range of concentrations used for the standard curve without the need to extrapolate. The PCR efficiency is close to 100% when the slope of the amplification curve is close to −3.32. See Section 5.3 for more information on PCR efficiency.

5.2.3 Template

There are several methods available for preparing material for a standard curve. A PCR product can be amplified from the target of interest, cloned or purified, and then serially diluted. Alternatively, to avoid the additional steps required for cloning a PCR product, long oligonucleotides (Ultramer™ Oligonucleotides), or gBlocks™ Gene Fragments, which will provide a known amount of product, can be purchased from IDT. Cloned amplicons, Ultramer Oligonucleotides, and gBlocks Gene Fragments provide ample product to create a 7 $\log_{10}$ standard curve. As a third option, a dilution series of cDNA can be prepared by reverse transcribing RNA from a tissue or cell line that expresses high levels of the target of interest. Pooled RNA from multiple cell lines is also commercially available. It is important to get as many data points as possible with at least 4–7 points for the standard curve to obtain a reliable estimate of the reaction efficiency.

Related Products at IDT

Ultramer™ Oligonucleotides and gBlocks™ Gene Fragments serve as excellent controls and, importantly, can be used as standards of known concentration. For more information and to order Ultramer Oligonucleotides and gBlocks Gene Fragments, visit the IDT website at www.idtdna.com.

Ultramer Oligonucleotides: IDT synthesis systems and chemistries allow high-fidelity synthesis of very long oligonucleotides (up to 200 bases). Suitable for demanding applications such as cloning, ddRNAi, and gene construction, Ultramer Oligonucleotides can save researchers a lot of time and effort because the entire fragment is directly synthesized by IDT.

gBlocks™ Gene Fragments: IDT offers double stranded DNA fragments up to 500 bp in size. gBlocks fragments are constructed using Ultramer Oligonucleotides and are sequence-verified.

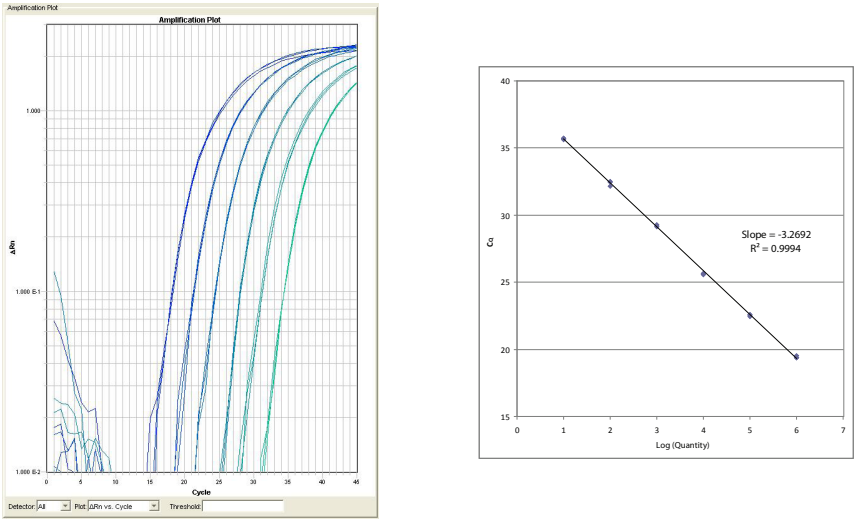


Figure 16. Range of Dilution Over Several Logs. A 6-point 10X dilution series over 5 orders of magnitude (1 x10^7 to 1 x 10^2 copies) was created for the JAK2 transcript. Amplification was performed using Gene Expression Master Mix (Applied Biosystems) on the Applied Biosystems 7900 Real-Time PCR instrument under standard cycling conditions: 2 min 50°C; 10 min 95°C; 45 x (15 sec 95°C, 1 min 60°C). **A**) The resulting amplification curves show a steady decrease in the threshold cycle with increasing sample concentration. For a reaction that is 100% efficient, the threshold cycle should increase by 3.3 cycles for each 10X dilution; i.e., it takes 3.3 cycles for the product to amplify10X. **B**) Linear regression of the amplification curve data derived from the dilution series generates a standard curve. For a reaction that is 100% efficient, the slope of the line should be –3.3 (for a standard curve generated from a 10X dilution series). When absolute standards are used (e.g., synthetic templates / long oligonucleotides), the y-intercept represents the C_q value for a single molecule.

5.3 PCR Efficiency

The efficiency of qPCR is influenced by many factors including target length, target sequence, primer sequence, buffer conditions, impurities present in the sample, cycling conditions, and the enzyme used [25]. The precision of a particular PCR assay is dependent upon the efficiency of the reaction. Current quantification models do not allow for efficiency correction between samples. However, assay-specific efficiency variation can be corrected for when calculating differences in gene expression. Such correction is required for obtaining the most accurate results. See Section 6.4.2 for more information on efficiency corrections.

The efficiency of a successful assay will be between 90 and 110%. Amplification efficiency can be calculated by analyzing the slope of the log-linear portion of the standard curve. When the logarithm of the initial template concentration is plotted on the X axis and C_q is plotted on the Y axis, PCR efficiency = $10^{-1/slope} - 1$ [1]. Based on this equation,

the theoretical maximum of 1.00 (100%) indicates a doubling of the product with each cycle. Efficiencies derived from dilution series are not exact values, but estimates with an uncertainty. This explains apparently impossible efficiencies of >100% and suggests that an assay with an efficiency of 94% is not really different from one with an efficiency of, for example, 99%. Hellemans et al. [26] presented formulas to determine the error (uncertainty) in the estimated PCR efficiency derived from a standard curve. Biogazelle qbase^{PLUS} software automatically calculates these errors (e.g., 94.0% +/-1.2%) and propagates this uncertainty in the final error bars for the results (see Section 8.3.2 for more information about Biogazelle software tools). The errors in the estimated PCR efficiency are generally smaller when using more dilution points or a wider dilution range.

5.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) is the lowest concentration at which 95% of the positive samples are detected [1]. The LOD indicates the concentration at which signal is lost, which is also where the measurements are no longer valid. To determine the LOD, examine the standard curve to find the most dilute sample that is still detectable in each case and with variance less than 1 C_q (see Section 6.3 for an explanation of C_q). Ideally, the no template control (NTC) reaction should not register a C_q value. However, if a C_q value is measured for the NTC reaction, the C_q of the most dilute test solution must be several C_q values lower than the C_q of the NTC to ensure reliability (a difference of 5 cycles would correspond to a contamination or background signal of 3%, which is below the precision of qPCR). Only C_q values that fall within the LOD are reliable. It is very important not to attempt to extrapolate C_q values that fall outside the lowest value.

The limit of quantification (LOQ) is the lowest amount of target that can be quantified with accuracy and reproducibility so that conclusions based on this data can be made with confidence.

5.5 Linear Dynamic Range

The linear dynamic range is the range over which a reaction is linear as established by the standard curve. This range is defined by the higher and lower limits of quantification (see LOQ in Section 5.4). Amplification measured outside this range cannot be quantified accurately with high levels of confidence. Ideally, the curve should span 5–6 $\log_{10}$ concentrations and, at a minimum, span 3–4 orders of magnitude with 4–5 data points. It is important to ensure gene expression calculations are made within the linear dynamic range.

5.6 Precision and Variability

The precision of an experiment depends on many factors. Results can vary with temperature changes, which cause annealing or denaturation, differences in concentration, pipetting errors, and stochastic variation [1]. Precision can be determined by performing sample replicates within an experiment and between experiments [1].

Sometimes normal biological variation between samples may exceed the variation in gene expression. Such variation must be taken into consideration when designing the experiment and analyzing the data. See Willems et al. [27] for examples of how to work with variable samples.

6. Data Analysis

This section provides general guidelines for qPCR data analysis. For assistance with data analysis, we recommend using a software program such as qbase^{PLUS} from Biogazelle (see Section 8.3.2 for more information).

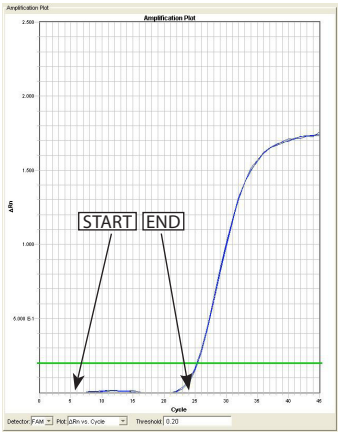
6.1 Rn, ΔRn, and RFUs

The reporter and the reference dyes (if used with your instrument) will have particular fluorescence emission intensities. The normalized reporter signal (referred to as Rn on some instruments) is the ratio of the fluorescence emission intensity of the reporter dye to that of the reference dye. A reference dye, ROX, is often used to normalize for any non-PCR–related fluctuations in the reaction. This reference dye is not required for all instruments, but is a helpful tool for troubleshooting. Instruments that do not use a reference dye often refer to the fluorescent signal as relative fluorescence units (RFUs). The reporter signal (Rn) minus the baseline fluorescence seen in early PCR cycles is referred to as baselined relative fluorescence units on some instruments and ΔRn on others. ΔRn or baselined RFUs plotted against cycle number generates the amplification curves and the C_q value.

6.2 Setting the Baseline

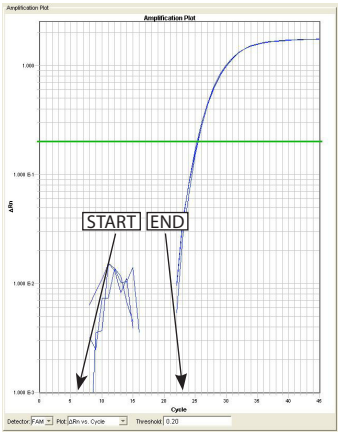
The baseline is the fluorescence present in the initial cycles of PCR prior to a detectable increase in signal resulting from accumulation of the amplicon. The baseline should be set in the normalized linear view (ΔRn vs. cycle) and should be wide enough to eliminate the background found in early cycles of amplification. However, it should not overlap with the area in which the amplification signal begins to rise above background (Figure 17). The baseline END value should be at a C_q value two cycles before the amplification curve for the highest expressing sample crosses the threshold. Setting the baseline is instrument-dependent. For more information on setting the baseline, please refer to the user manual for the instrument you are using.

Linear View



Panel A. Linear Plot.

Log View



Panel B. Log Plot.

Figure 17. Plotting Amplification Data.

6.3 Setting the Threshold

The threshold is the ΔRn level that determines the threshold cycle, or C_q. The threshold is set on the baseline, corrected from relative fluorescence. In Figure 18 it is seen in log view as a line above the baseline and through the amplification curve in the exponential growth region (linear increasing signal in a log scale). The C_q is the cycle number at which the amplification plot crosses the threshold [3]. Setting the threshold is instrument dependent. For more information, refer to the user manual for the instrument you are using.

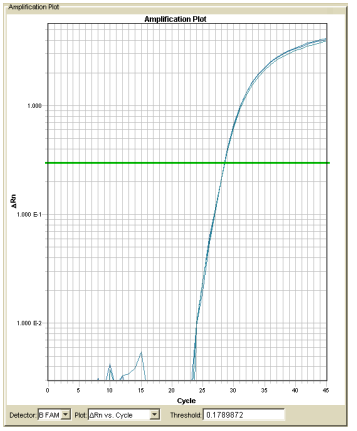


Figure 18. Proper Threshold Setup. The threshold is indicated by a green line through the curve in the exponential region.

6.4 Determining Gene Expression Changes

Gene expression is quantified by the number of cycles required for fluorescence signal to reach the threshold. During amplification, the fluorescence from a more highly expressed gene will reach the threshold at an earlier cycle than a gene with a lower level of expression. To analyze the amount of PCR product, investigators compare either absolute levels of RNA copies for each sample or relative levels of RNA to an unchanged control [8].

6.4.1 Absolute Quantification

Absolute quantification requires generating an absolute standard curve for the gene of interest and calculating the number of gene copies in an unknown amount of sample from a comparison with the standard curve. The standard curve can be created using a known amount of DNA, linearized plasmid, PCR amplicon, or oligonucleotide. The unknown "test sample" amount can then be interpolated from the standard curve calculation. The reliability of this method is dependent on identical amplification efficiencies of the known and test samples [28], and on the accuracy with which the standard samples are quantified.

6.4.2 Relative Quantification

Relative quantification is the more commonly used method of analysis and is expressed as the fold difference in gene expression between test and control samples for a given gene. Importantly, relative quantification cannot be easily used to compare expression levels between genes (due to the assay-dependent relationship between C_q value and input amount). To normalize input quantities, expression of the target gene of interest is typically compared to one of the following: an endogenous control (reference gene), an exogenous control, a reference gene index, or a target gene index [28]. Relative quantification measures the difference (Δ) between the C_q values [28]. The equation used depends on the similarity or differences in the efficiencies of the reactions and the number of reference genes being used [26].

6.4.2a Normalization

Normalization corrects for inequalities in DNA concentrations resulting from variations during sample preparation to enable comparison across samples. Typically, normalization uses one or more reference genes as an internal control for this comparison. However, samples may also be normalized to sample size, total RNA, or to an artificial molecule included in the reaction [29]. Samples that are being compared must have been subjected to identical sampling, isolation, reverse transcription, and qPCR conditions. In addition, the results obtained depend on the quality of the normalizer chosen—so verifying a representative normalizer that has identical expression across all samples tested is vital for the accuracy of the results.

• Normalizing to a reference gene

When normalizing to a reference gene, it is very important that the reference gene is experimentally validated to ensure that it is an accurate measure against which to compare all other sample variations. Ideally, more than one reference gene should be tested. A pilot study can be conducted to select the best set of reference genes out of a series of candidates. Analysis of reference gene stability can be performed with tools such as geNorm [30] and qbase^{PLUS} [26]. The reference genes should have stable mRNA expression and the amount of reference gene mRNA should be strongly correlated with the total amounts of mRNA in the samples [1]. When using this method, it is critical that the reference genes used do not vary with experimental conditions. Normalized data is reported as a ratio of the mRNA concentration of the gene of interest to the mRNA concentration of the reference gene [1]. This can be calculated by a comparative C_q method or a standard curve method.

• No efficiency correction

This method is based on the assumption that all the reactions occur with 100% efficiency so that there is a doubling of the target DNA with each cycle of PCR. Such conditions are unlikely with multiple assays, and therefore, we do not recommend this method. Small differences in PCR efficiencies between targets and reference genes can lead to false expression ratios and distort relative expression measurements. The effect of differing PCR efficiencies becomes more apparent with increasing cycle number.

$$\Delta C_q = C_q \text{ target} - C_q \text{ reference} \qquad \textit{equation (1)}$$

$$\Delta\Delta C_q = \Delta C_q \text{ sample} - \Delta C_q \text{ control} \qquad \textit{equation (2)}$$

$$R = 2^{-\Delta\Delta C_q} \qquad \textit{equation (3)}$$

1. Determine the C_q value for each reaction.
2. Calculate the difference in C_q values for the gene of interest (target) and the endogenous control(s) (reference). This is the ΔC_q as shown in equation (1).
3. Subtract the control-condition ΔC_q from the treated-condition ΔC_q to find the $\Delta\Delta C_q$ as shown in equation (2).
4. To calculate the ratio of gene expression change (R) of the sample relative to the control, the negative value of this subtraction ($-\Delta\Delta C_q$) becomes the exponent of 2 and represents the difference in the "corrected" number of cycles to threshold. The value of 2 is used because the assumption is that the product doubles in each cycle. This is shown in equation (3).
5. For example, if the control sample ΔC_q is 2 and the treated sample ΔC_q is 4, then $2^{-\Delta\Delta C_q}$ will be 0.25. Therefore, the level of the gene of interest in the experimental sample is 25% of that in the control sample. This can be verified by looking at the qPCR curves—the experimental sample would require additional cycles to produce the same amount of product as the control sample because of the lower starting amount of the transcript being measured [8, 28, 31].

6.4.2b Efficiency-Corrected Gene Expression Measurements

To account for gene expression efficiency differences, one of the following equations should be used. This is the recommended method for calculating fold changes in gene expression because it takes into account variation in assay efficiency [28]. In equations 4–6, E represents the fold change per cycle per gene. For example, for an assay with 95% efficiency, E would be 1 + 0.95, or 1.95 (E is also called the base of the exponential amplification).

- ***Efficiency Corrected based on one sample***

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_q \text{ target (control - sample)}}}{(E_{ref})^{\Delta C_q \text{Ref(control – sample)}}}$$

equation (4)

- ***Efficiency Corrected based on multiple samples***

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_q \text{ target (MEAN control - MEAN sample)}}}{(E_{ref})^{\Delta C_q \text{Ref(MEAN control – MEAN sample)}}}$$

equation (5)

- ***Efficiency Corrected based on multiple samples and multiple reference genes***

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_q \text{ target (MEAN control - MEAN sample)}}}{(E_{ref\ index})^{\Delta C_q \text{Ref index(MEAN control – MEAN sample)}}}$$

equation (6)

Example

A researcher used BRCA1 as a target gene and GAPDH as a reference gene. After performing a standard curve with a set of plasmid positive controls, the efficiency of each assay was calculated from the slope of the standard curve:

BRCA Assay Efficiency = 93%
GAPDH Assay Efficiency = 98%

The researcher ran the control and treated samples in triplicate which resulted in the following C_q values:

		C_q		Mean C_q	
		BRCA1	GAPDH	BRCA1	GAPDH
Control C_q	Replicate 1	25.2	14.1	25.4	14.0
	Replicate 2	25.7	13.8		
	Replicate 3	25.4	14.2		
Treated C_q	Replicate 1	22.1	13.5	22.3	13.7
	Replicate 2	22.5	13.9		
	Replicate 3	22.3	13.7		

Table 7. C_q Values for Determining Relative Quantification.

Using equation (4), the researcher was able to calculate the gene expression fold change between experimental and control samples.

$$\Delta C_q\_BRCA1 = C_q \text{ control} - C_q \text{ treated}$$
$$\Delta C_q\_BRCA1 = 25.4 - 22.3$$
$$\Delta C_q\_BRCA1 = 3.1$$

$$\Delta C_q\_GAPDH = C_q \text{ control} - C_q \text{ treated}$$
$$\Delta C_q\_GAPDH = 14.0 - 13.7$$
$$\Delta C_q\_GAPDH = 0.3$$

$$R = \frac{(1+0.93)^{3.1}}{(1+0.98)^{0.3}}$$

$$R = 6.25$$

Therefore, in the experimental sample, BRCA1 expression levels increased 6.25-fold relative to the control sample. A more accurate measurement was made by taking the amplification efficiency of each assay into account. If equation (3), which assumes 100% assay efficiency, had been used instead, the result would have been 6.96, which would have been an overestimation of the fold change in gene expression.

This example is a simplistic analysis that uses a single reference gene. In practice, at least 2 stably expressed reference genes are recommended. For a more detailed outline, see Hellemans et al [26].

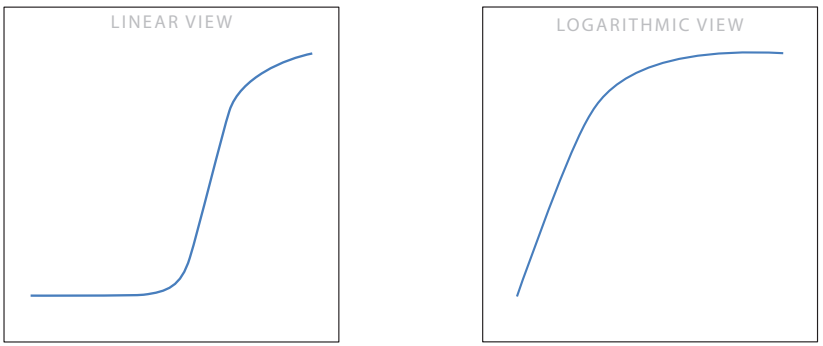
6.5 Qualitative Analysis

For some applications, the purpose of PCR may be to determine the presence or absence of a particular nucleic acid target rather than to compare mRNA levels among samples. For these applications, it is still very important to assess the quality of the experiment—particularly to determine its sensitivity. A target may appear to be absent when it is actually present in a low amount because it is not detected due to the experimental setup.

7. PrimeTime® qPCR Assay Troubleshooting

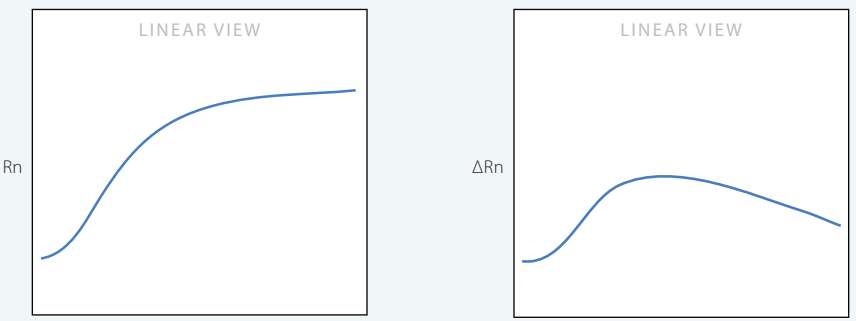
As qPCR is a complex, multifaceted process, suboptimal amplification may be observed for a number of reasons. Troubleshooting or generation of additional data may be required to achieve optimal results. Stylized examples of the types of problematic qPCR data that can be encountered are shown following. Match your data with one of these and refer to the indicated section to learn what causes such curves and how to remedy them.

Good qPCR curves should look like these:



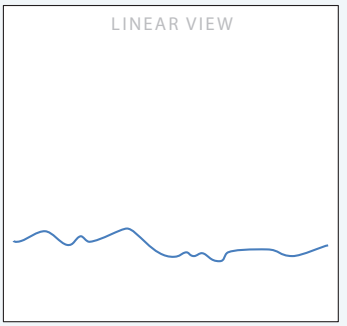
Problem qPCR Curves

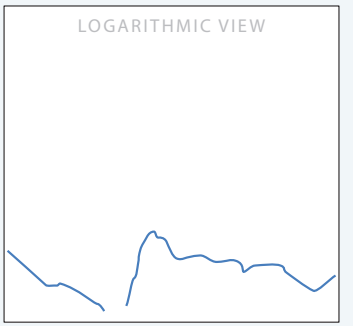
Your curves look like this:



You likely have too much template—***See Section 7.4.4***.

Your curves look like this:



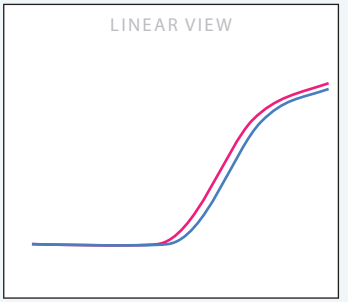


There is no amplification—***See Section 7.1.***

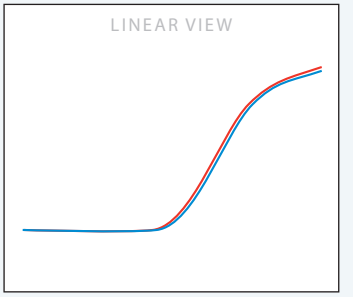
Several factors can contribute to this:

- No target in sample
- Missing reaction component
- Sample degradation
- Incorrectly assigned dye detector
- Poor assay design

Your replicates look like this:



Instead of this:

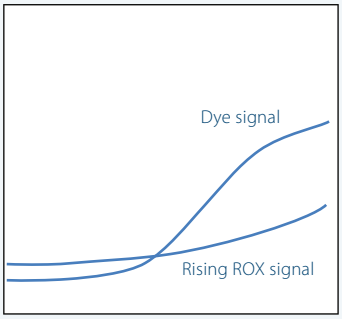


Inconsistent replicates—***See Section 7.6***.

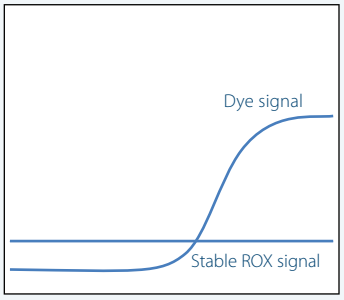
Several factors can contribute to this:

- Pipetting errors
- Thermal calibration of thermal cycler
- Low target copy number
- Inappropriate cycling conditions

Your curves look like this:



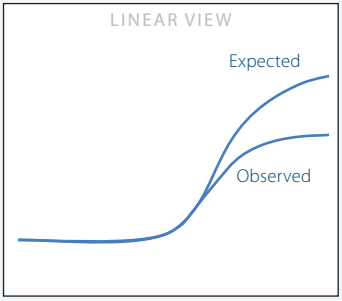
Instead of this:



You are likely experiencing sample evaporation, resulting in rising ROX concentration.

See Sections 7.8.1 and 7.8.3

Your curves look like this:

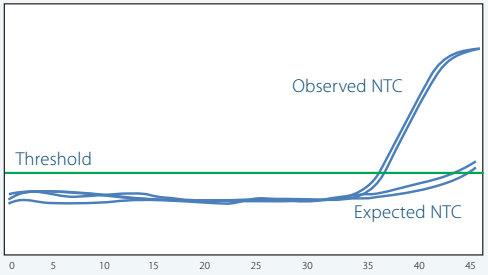


Low height of amplification curve.

You may be experiencing:

- Dye quenching by proximal G base—***See Section 4.2.2b***.
- Differences in probe concentration—***See Section 7.2.2***.
- Too much ROX in your sample—***See Section 7.8.1***.
- Differences in master mixes used—***See Section 4.3.2a***.
- Signal bleed over—***See Section 7.4.1***.
- Inherent differences in fluorescence intensity of different dyes—***See Section 7.2.5***.

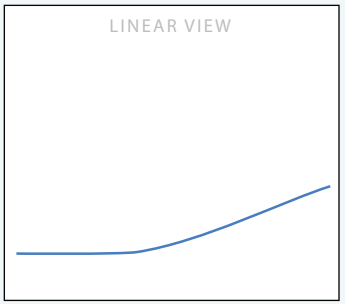
Your curves look like this:



You are observing true amplificaton in the No Template Control (NTC).

See Section 4.2.6b

Your curves look like this:

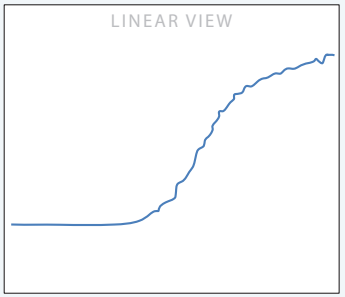


The reaction has poor efficiency—***See Section 7.3.***

Several factors can contribute to this:

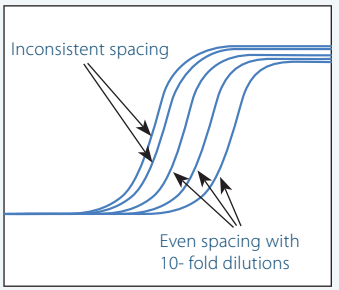
- Poor primer design
- Low fluorescent dye intensity and poor instrument optics
- Sample inhibition
- Incorrect primer concentration

Your curves look like this:



- You likely have too much probe or insufficient reference dye in your reaction—***See Section 7.5***
- You have noisy data.

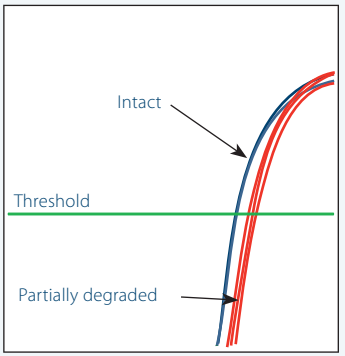
Your curves look like this:



Your initial dilutions (higher concentrations) for the standard curve are not producing expected C_q intervals.

See Section 7.6.4

Your curves look like this:



You are observing later than expected C_q values and/or inconsistent replicates.

You may be experiencing:

- Nucleic acid degradation—***See Section 7.6.4***
- Pipetting errors—***See Section 7.6.1***

7.1 Little to No Amplification

7.1.1 Reaction Setup

Omitted or incorrectly diluted reaction component. It is possible that one of the components was inadvertently left out or added at an incorrect concentration. We recommend that you repeat a failed experiment once to make sure it was not due to a simple mistake at the first attempt. Refer to the protocol to verify that all listed components were added and at the appropriate concentration. See Section 4.3.2 for a protocol.

Expired reagent. Check the expiration date on all reaction components to make sure they are not expired. Replace any expired reagents.

Incorrect instrument settings. The baseline fluorescence should be above background. The absence of fluorescence signal may be due to a problem with the instrument, such as an incorrect filter setting. Run a reaction without the primer/probe mixture and compare it to an experimental plate to see if the experimental plate is producing any fluorescence above background.

7.1.2 Reaction Parameters

Suboptimal amplification can be improved by adjusting the reverse transcription reaction and/or the PCR cycling conditions.

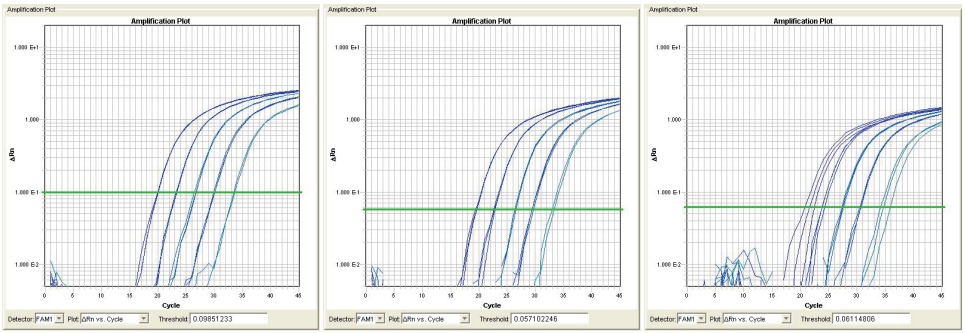
Nonoptimal reverse transcription conditions. Check the following parameters:

> ***Length:*** Change the length of the reverse transcription step in 5-minute increments up to a maximum of 60 minutes.
>
> ***Temperature:*** The reaction should be set up on ice so that cDNA synthesis does not begin prematurely. Change the temperature of the reverse transcription reaction in 5°C increments up to a maximum as determined by the enzyme and the type of primer you are using.

Nonoptimal PCR conditions. Check the following parameters:

> ***Annealing and extension times:*** Figure 19 shows the effect of different annealing/extension times on qPCR amplification. Annealing or extension steps that are too short can result in little or no amplification. When the annealing step is too short, amplification can be inconsistent or decreased, or may not occur at all. As a general

recommendation, adjust the time in 15-second increments up to a maximum of 1 minute for amplicons that are 250 bp or shorter when using standard cycling conditions; for fast cycling master mixes, use 3-second increments. Refer to the user guide for your master mix for more specific recommendations.



A. 60 Sec Anneal/Extend. B. 45 Sec Anneal/Extend. C. 15 Sec Anneal/Extend.

Figure 19. Assay Results When Run at Different Cycling Conditions. Reactions were run in 384-well plates using the Applied Biosystems Gene Expression Master Mix, 10 µL per reaction, and a 5 log dilution of Human Universal Reference RNA cDNA. When the anneal/extend step becomes too short (C), amplification is inconsistent and decreased or even prevented.

Annealing temperature: An annealing temperature that is too high can result in no amplification, while one that is too low can result in nonspecific amplification or amplification of primer-dimers. Change the annealing temperature in 2°C increments.

Cycle number: Too few reaction cycles can result in little or no amplification. The maximum number of cycles varies from 35 to 45 but the ideal number of cycles is 40. Most researchers disregard C_q values greater than 38 because amplification beyond cycle 38 is indicative of inefficient PCR.

7.1.3 Primer or Probe Integrity

Primer problems. Determine whether reduced or absent fluorescence signal is due to inefficient amplification by the primers. First, check that the primers (or assays) were diluted correctly. Next, rerun an assay that has worked previously to rule out problems with the master mix or thermocycler. If the problem is assay specific, run a sample of the reaction product on a high-resolution agarose or non-denaturing acrylamide gel to see if a PCR product of the correct size is present. If so, the primers are likely working. See Section 4.3.2 for a protocol.

Probe degraded or degrading during reaction. See Section 7.7 *High or Variable Background*.

Nuclease contamination causing degradation of nucleic acids. Use a reagent such as the IDT RNaseAlert or DNaseAlert Kit to check for nuclease contaminants. IDT recommends that the probe be resuspended in nuclease-free TE buffer (10 mM Tris pH 7.5–8.0, 0.1 mM EDTA) and stored in aliquots at –20°C. See Section 4.3.1 for a resuspension protocol and Section 2.4 for more information on avoiding nuclease contamination.

7.1.4 Sample Expression

Target below the limit of detection. Amplification signal will not be detectable if the target gene is absent or expressed below the limit of detection (LOD) for the assay. To determine the LOD for a particular assay, amplify a positive control across a range of concentrations from 1 to 100,000 copies per well. Optimization of the reaction may be required to achieve low LOD. Guidelines for optimization are provided in the protocol in Section 4.3.2. IDT suggests using a linearized plasmid that contains the target sequence as template because it can easily be quantified and amplified. The target sequence can be ordered as a gBlocks™ Gene Fragment or an Ultramer™ Oligonucleotide from IDT (see the Products Box (end of Section 5.2.3) for more information).

When the reaction has been optimized and the LOD is known, try amplifying the cDNA/DNA using 10–100 ng cDNA per reaction. If the DNA still does not amplify, try a new cDNA/DNA template preparation to rule out transcription or sample prep inefficiencies. Also, using a gene-specific RT primer in the first-strand cDNA synthesis can also be used to increase the amount of target for detection.

Not enough template. If too little template was added to the reaction, the polymerase may not have been able to amplify the target to a level above the limit of detection

(LOD) of the assay. Increase the amount of template in the reaction. Preamplification of RNA, selecting for poly(A+) RNA, or using a gene-specific RT primer in the first-strand cDNA synthesis can also be used to increase the amount of target for detection. See Section 4.2.1 for more information.

Related Products at IDT

RNaseAlert™ and DNaseAlert™ Kits: These reagents are fluorescence-quenched oligonucleotide probes that emit a fluorescent signal only after nuclease degradation and allow for rapid, sensitive detection of RNases or DNases. For more information and to order these products, visit the IDT website at www.idtdna.com.

7.2 Low or Delayed Signal

7.2.1 Design Specificity

The C_q is higher than the expected value. This observation likely indicates low primer efficiency. There may be mismatches between the target and primer/probe sequences. Determine the amplification efficiency by using a serial dilution of a plasmid containing the target sequence. Perform a BLAST search to confirm the specificity of the target and assay sequences. See Section 8.3.1 for more information about this free NCBI tool. Run a gel to see if the correct size of product is being amplified. If you are not using a predesigned assay from IDT, check that your primer and probe do not span a SNP site. Note that all PrimeTime qPCR Assays are tested with up-to-date sequence information specifically to avoid assay design over SNP sites, thus preventing this problem.

7.2.2 Reaction Setup

Primers or probe were not completely resuspended. See Figure 22 for an example of curves from a reaction run with a low concentration of probe. Suboptimal primer or probe concentration is most often due to incomplete resuspension. Check the resuspension protocol in Section 4.3.1 and confirm that these reagents were properly resuspended. Calculation or dilution errors can also result in too little probe/primer. A less likely possibility is that the recommended primer and probe concentrations (Section 4.3.2) are not optimal for your sample. This can be tested by adjusting the concentration of primers and probe in 25 nM increments.

7.2.3 Sample Expression

Inhibitors in reaction. Running several dilutions of the cDNA/template can be used to determine the presence of inhibitors that may be limiting target amplification. If increasing template/cDNA concentration does not lead to a linear increase in signal it is likely that inhibitors are present in the sample. Also, if there are inhibitors present in the sample, the highest template concentration will contain the highest concentration of inhibitors. Amplification efficiency will be reduced and 10X dilutions will not be separated by 3.32 cycles. Make a new preparation or repurify the cDNA/template if this is the case.

Sample prep method skews target representation. Due to inconsistencies in sample isolation methods, cDNA preparations can have differing amounts of transcript ends. Multiple assays for the same gene should result in the same C_q values, validating the true expression level of that gene under the specific experimental conditions.

7.2.4 Baseline

Baseline set incorrectly. The baseline is a critical component for determining accurate C_q during qPCR data analysis. If set wrong, amplification results can appear reduced or delayed. The baseline should be wide enough to eliminate background that occurs in early cycles of amplification, but its end value should occur prior to the change in fluorescence and before the amplification curve crosses the threshold (Figure 20). Never start the baseline at cycle 1. Default should be 3, but you should always check that the baseline is stable when selecting the start location. As depicted in Figure 20, setting the baseline incorrectly can result in either delayed C_q or sloping traces that also have a delayed C_q value. See Section 6.2 for more information on setting the baseline.

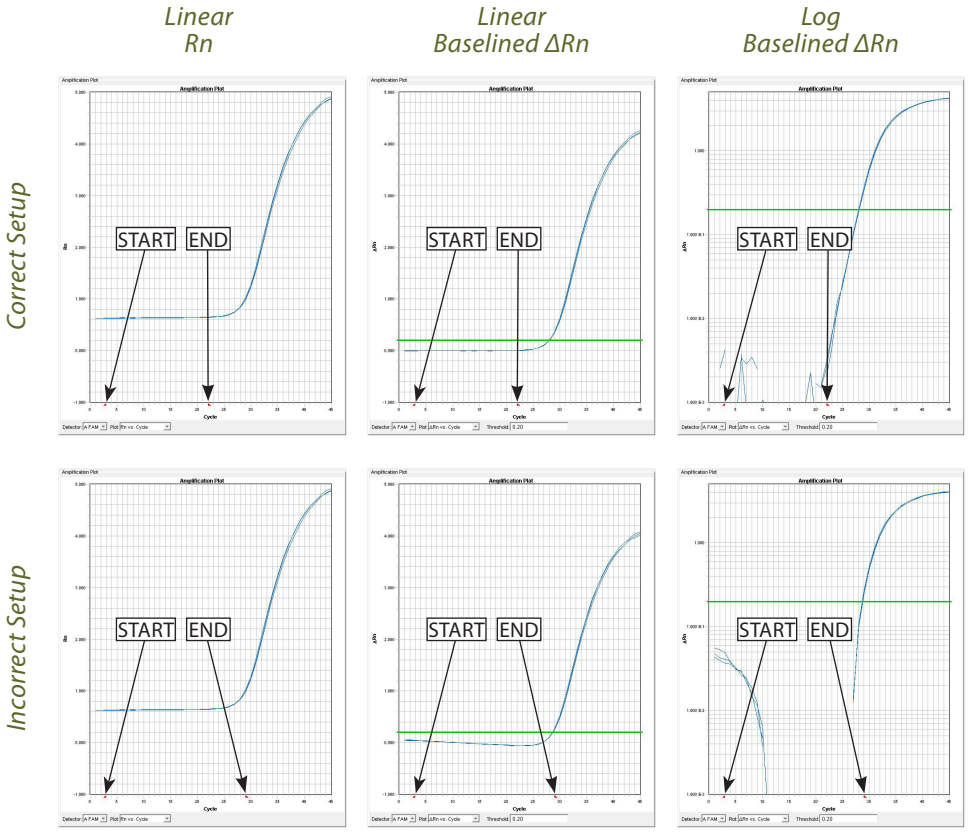


Figure 20. Correct and Incorrect Setup of the Baseline. Baseline start and stop values in panels A–C are set correctly, while those in panels D–F are set incorrectly, with the END point of the baseline setting extending into the exponential part of the curve. Incorrect settings result in either delayed C_q values (D) and (F) or sloping traces that also have a delayed C_q value.

7.2.5 Choice of Dye(s)

Inherent differences in fluorescence intensity of different dyes. The height of the amplification curve may vary with the reporter dye being used and the optical capability of your instrument. The magnitude of fluorescent intensity is a function of both the intrinsic properties of the compound (such as quantum efficiency and molar absorptivity) and the incident radiant power. Therefore, using a dye of low fluorescent capability on an instrument that has not been calibrated for that dye may result in reduced height of the amplification curve.

7.3 Poor Efficiency

7.3.1 Design Specificity

C_q higher than expected value. This observation likely indicates low primer efficiency. There may be mismatches between the target and primer/probe sequences or non-optimal PCR conditions. Perform a BLAST search to confirm the specificity of the target and assay sequences. See Section 8.3.1 for more information about this free NCBI tool. Run a gel to see if the correct size of product is being amplified. If you are not using a predesigned assay from IDT, check that your primer and probe do not span a SNP site. Note that all PrimeTime qPCR Assays are designed with up-to-date sequence information specifically to avoid assay design over SNP sites, thus preventing this problem.

7.3.2 Reaction Setup

Efficiency of the reaction has decreased over time. Rerun an assay that has worked previously to see if the problem is assay-specific. If so, assay degradation may have occurred. Reorder the assay, if necessary. If the decrease in efficiency is not assay specific, it is possible that the master mix was improperly stored or that the template has degraded. Check the instructions for the master mix to verify proper storage. Also check for expiration dates to be sure the mix is still current. Replace any expired reagents. Make a new preparation of the template if necessary.

Incorrect primer concentration. Check that the tubes containing the primers were spun down before resuspension and the contents diluted to the correct concentration. Increase the concentration of primers in 50 nM increments and monitor the shape of the amplification curve for any improvement.

7.3.3 Instrument

Cycling temperatures or time parameters incorrectly set. It is possible that the cycling temperatures and time parameters are not set correctly. Check that parameters match the recommended cycling conditions for the master mix used. Do not interchange cycling protocols between fast and standard master mixes. Guidelines are provided in the protocol in Section 4.3.2. Also, make sure the fluorescence is being collected at the extension step.

7.4 Excessive or Unexpected Signal

7.4.1 Instrument Calibration

Signal detected in wrong channels. There may be bleed over of dye signal into another channel making it difficult to obtain an accurate reading of signal for any given dye. This is usually due to improper instrument calibration. Ensure that the real-time PCR instrument is compatible with and calibrated for each of the dyes used in a qPCR experiment. To determine if calibration of your instrument is required, run assays with each dye individually and use the software to determine whether dyes other than that expected show up in a particular channel (e.g., run an assay with a Cy5 labeled probe and look at the other dye channels to see whether there is bleed-over signal). Erroneous signal in a channel is indicative of poor calibration. To calibrate your instrument, refer to the manufacturer's protocol for your instrument.

All instruments should have monthly maintenance, including calibration. Refer to the instrument manual for instructions. Run an assay that has previously worked to see if it is still working.

7.4.2 Assay Specificity

Non-specific assay. Unexpected or excessive expression may mean the assay is not specific for the target transcript or is detecting additional transcript variants (e.g., alternatively spliced forms). Using a second PrimeTime Predesigned qPCR Assay located in a different region of the target will verify the results or uncover an artifact.

7.4.3 Contamination

Reagent Contamination. It is possible that the master mix or other reagents have been inadvertently contaminated with the amplicon. See page 19 for additional ways to prevent contamination.

Genomic Contamination. Amplification occurring in the no RT control may indicate genomic contamination. This may also result in higher than expected expression in your samples. If genomic DNA is not your template of interest, treat samples that may contain genomic DNA contamination with DNase prior to cDNA synthesis (see Section 2.1). When possible, design primers, probes, or amplicons to span an exon–exon junction to avoid amplification of genomic DNA (see Section 4.2.1).

7.4.4 Template Concentration

Addition of too much template. The template concentration is too high if C_q values earlier than 15 are obtained. The addition of too much template may cause miscalculation of the baseline factor and affect the shape of the curve. Dilute the template as necessary to ensure that C_q values are greater than 15.

7.5 Noisy Data

Addition of too much probe. High probe concentration is usually due to errors in dilution or reaction setup. Refer to the protocol to verify that probe was added and at the appropriate concentration. See Section 4.3.2 for a protocol. Also ensure that calculations for the dilution of probe are correct, and that the probe was diluted appropriately. Less likely is that the recommended primer and probe concentrations are not ideal for your sample. This can be tested by decreasing the concentration of probe stepwise by 25 nM.

Insufficient reference dye. ROX degradation or low ROX concentration can cause the ΔRn to be high and noisy as the instrument tries to normalize data using a weak, inconsistent ROX signal. See 7.8.2 for more details.

7.6 Inconsistent Replicates

As a general guideline, replicates should not vary by more than $0.5C_q$; however, this can be more stringent based on he differences in the samples being analyzed.

7.6.1 Reaction Setup

Poorly mixed reaction. This is a common mistake that can result in a large spread in the replicates and irregular spacing between dilutions of the standard. Be sure to gently mix the sample after all components are added.

Master mix not used. Every single sample should be treated in the same manner in order to achieve reliable results. For that reason, it is very important to use a master mix to ensure that every sample receives the same amount of each of the reaction components. See Section 4.3.2a for more information on master mixes.

Pipetting errors. Ensure that your pipettes are properly calibrated and that the seals are in good repair. Use pipette tips once only and ensure other good pipetting techniques.

7.6.2 Reaction Parameters

Activation step not long enough. It is possible that the PCR activation step was not long enough for the enzyme used. Some hot start enzymes require longer activation at 95°C than others. Check the requirements for the enzyme you are using.

7.6.3 Instrument Calibration

Temperature calibration needed. If, for example, replicates are inconsistent or the C_q difference between successive 10X dilutions is not 3.32, the instrument may require temperature calibration. This is likely if results are inconsistent across the block. Try repositioning the samples that are giving the delayed signal into different wells to see if results improve. If so, it suggests that the block is not heating uniformly and the real-time PCR instrument requires a temperature calibration.

Thermal cyclers usually require calibration for temperature consistency once every 6 months. Most instruments have a built-in test run, or self-check protocol, that allows the instrument to recalibrate itself if determined necessary. You can also use temperature-sensitive dyes (Life Technologies), compatible with most instruments, to calibrate the thermal cycler. Determine whether your instrument has a built-in temperature calibration self-check protocol.

New light source needed. If the instrument has been recalibrated for the dyes used but samples continue to provide inconsistent results when tested in different wells, it is possible that the instrument needs a new light source. Refer to your instrument manual for instructions on how to replace a light source/bulb.

7.6.4 RNA Sample Quality

cDNA replicates yielding variable C_q values. It is possible that during RNA sample preparation the quality of some samples was compromised, e.g., by degradation, which is reflected in the cDNA product. Figure 21 shows how degraded template can affect qPCR amplification. Poor template quality can be due to varioius factors, including the RNA isolation method, poor reverse transcription, and improper storage and handling. Check sample quality by assessing the RNA integrity with a system such as Experion (BioRad) or 2100 Bioanalyzer (Agilent) or by examining a small amount on a gel (see Section 2.3). Use TE buffer to resuspend the RNA sample. Check the RT reagents for contamination or expiration.

Initial dilutions of standard curve not producing expected C_q intervals. Contaminants may be present in the sample. These can originate from the host tissue or cell. They can also be present in enzymes used in the RT reaction or qPCR, components used to isolate the RNA, or other reagents added in the process. Such contaminants can inhibit the amplification of the sample. Inhibition by contaminants is often more pronounced in the least diluted standards, when the contaminants are still fairly concentrated.

To check for inhibitors, include a serial dilution of your sample in an endogenous control assay. The highest concentration of template contains the highest concentration of inhibitor, which causes a delayed C_q. In contrast, a lower concentration contains less inhibitor, resulting in an earlier C_q and a change in the slope.

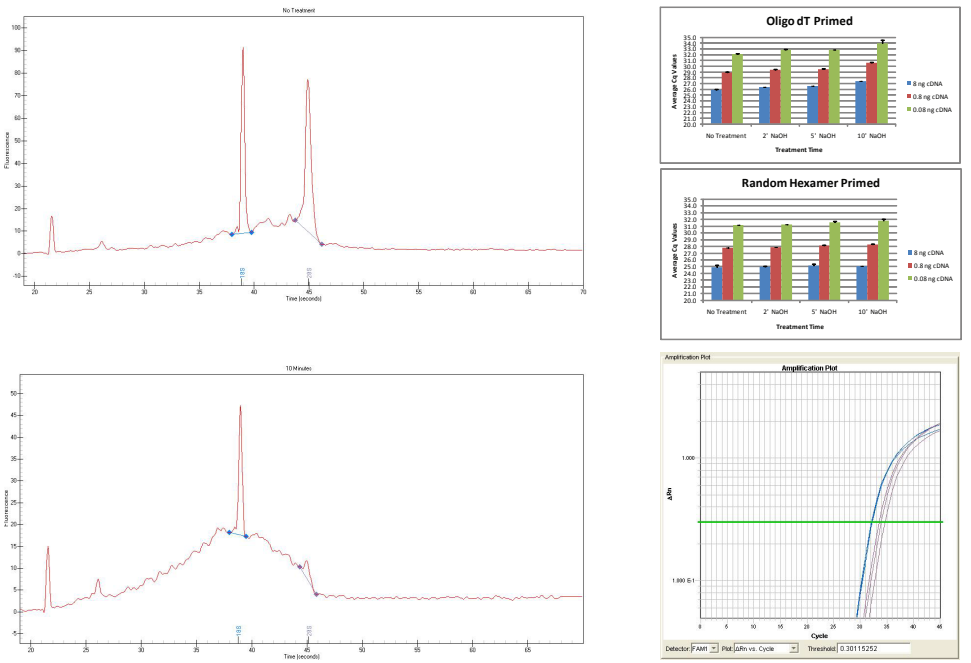


Figure 21. Poor Sample Quality Delays C_q. To demonstrate the effect of poor RNA quality on C_q values, 8 ng, 0.8 ng, and 0.08 ng RNA were left untreated (intact) or were treated with NaOH for 2 min, 5 min, or 10 min to create increasing levels of degradation. The electropherograms show samples of (A) intact RNA and (B) degraded RNA. C_q values were delayed for the degraded RNA, compared to the intact RNA sample, as shown by (C) the average C_q values and (D) the amplification curves.

7.7 High or Variable Background

7.7.1 Reaction Setup

Addition of too much probe. High probe concentration is usually due to errors in dilution or reaction setup. Refer to the protocol to verify that probe was added and at the appropriate concentration. See Section 4.3.2 for a protocol. Also ensure that calculations for the dilution of probe are correct, and that the probe was diluted appropriately. Less likely is that the recommended primer and probe concentrations are not ideal for your sample. This can be tested by decreasing the concentration of probe stepwise by 25 nM.

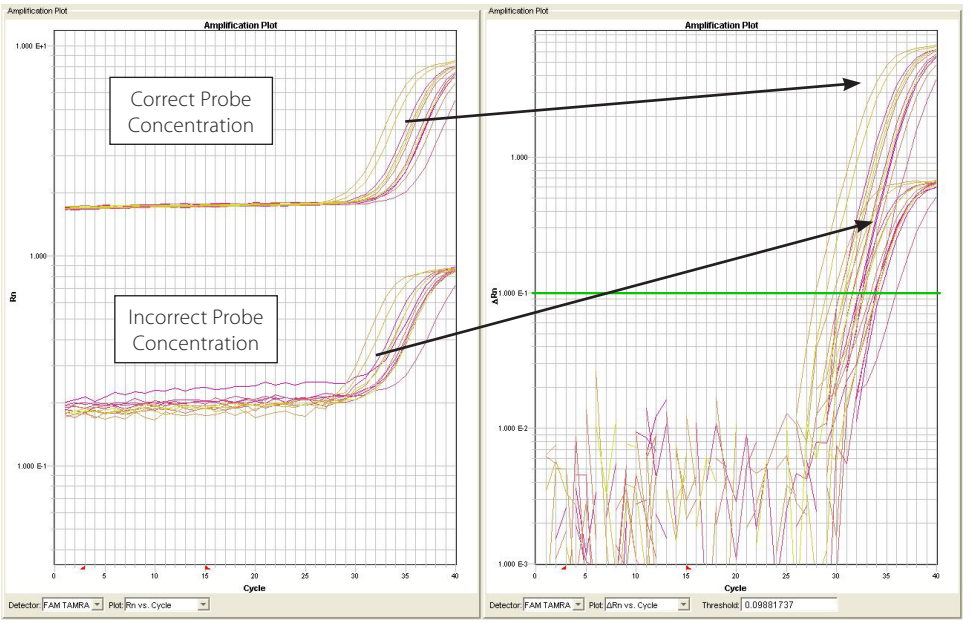


Figure 22. Low Signal Due to Incorrect Probe Concentration (R_n Linear View).

7.7.2 Primer and Probe Integrity

Inadequate probe purification. High background can also result from poor purification of the probe. IDT performs HPLC, mass spectrometry, and capillary electrophoresis on each probe synthesized to ensure probe integrity and purity. These QC data are all posted on the customer's web account at no extra charge.

Probe degraded prior to cycling. Degradation of the probe at the start of the experiment will be indicated by extremely high background fluorescence that does not change with cycling. This is especially likely if the background fluorescence has changed since a previous run with the same probe. If the probe has degraded, you will need to use a new aliquot, if available, or a newly synthesized probe. IDT recommends that the probe be resuspended in TE buffer (10 mM Tris pH 7.5–8.0, 0.1 mM EDTA) for storage. Water is a poor choice of solvent as it can be acidic and cause depurination and strand scission. Always store probes in aliquots at –20°C, protected from light. See Section 4.3.1 for a resuspension protocol.

Probe degrading while cycling. Run a reaction with the probe alone (no primers) to see if the signal increases. If there is a significant increase in fluorescence, it is likely that the probe is being degraded during cycling due to the presence of contaminating nucleases. Also, be sure to run a no-template control containing probe and primers alone; this should show low flat line fluorescence or slightly increasing fluorescence. Follow good laboratory practices to avoid introducing nuclease contamination.

Poor quenching. High background can also be caused by poor quenching of the probe. Probes longer than 30 bases with a single quencher at one end may have poor quenching ability. Design probes to be shorter than 30 bases, unless using double-quenched probes (see below). If there is a long run of As or Ts, the addition of LNA bases can help raise the T_m. Poor quenching may also occur if an inappropriate quencher is being used for the assay. Verify that the quencher and fluorophore are a good pair. See Section 4.2.2b for proper design of probes and Table 3 for absorbance ranges of quenchers and fluorophores.

IDT offers dual-quenched probes that contain a ZEN quencher internally in addition to a quencher on the 3' end of the probe. Such dual-quenched probes can resolve problems associated with poor quenching by significantly decreasing background and increasing signal intensity. The presence of an internal quencher also makes the use of longer probes possible. See Section 4.1.3 for more information.

Probe degradation. A degraded probe should exhibit constant fluorescence. To check probe integrity, perform a signal to noise ratio (STNR) assay. Dilute an aliquot of the probe to a final concentration of 0.25 µM. Add 1U micrococcal nuclease and digest the sample at 37°C. Measure the increase in fluorescence over a background reaction of probe plus buffer without micrococcal nuclease. An intact probe should exhibit increased fluorescence.

7.7.3 Instrument

Fluorescent contaminants. It is possible that the thermal block contains fluorescent contaminants resulting in high background and decreased signal. Run a background or water plate to confirm the background is still within specification. Refer to the instrument manual or contact a service organization.

7.8 Passive Reference Problems (only applies to instruments that use ROX dye)

The signal of the passive reference should be significantly higher than the background signal of the instrument. Check your master mix to see if it has the correct passive reference concentration (high or low ROX) for the instrument that you are using. The use of high or low ROX will depend on the instrument—refer to the instrument manual for the appropriate concentration.

7.8.1 Lower than expected amplification curves—high ROX.

If the ROX signal is too high, the ROX signal will be higher than FAM in the multicomponent plot and the ΔRn will be low (Figure 23). If samples are not sealed properly, the effective ROX concentration measured by the instrument increases due to evaporation, which increases the number by which your samples will be normalized and results in incorrect C_q values.

7.8.2 Higher than expected or noisy amplification curves—low ROX.

ROX degradation or low ROX concentration can cause the ΔRn to be high and noisy as the instrument tries to normalize data using a weak, inconsistent ROX signal. See Figure 23.

7.8.3 Amplification curve drops off and has an atypical shape

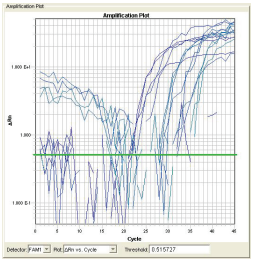
Evaporation. Passive reference signal fluctuation can be caused by evaporation if sample tubes or plates are not sealed properly. Ensure secure sample tube/plate closure.

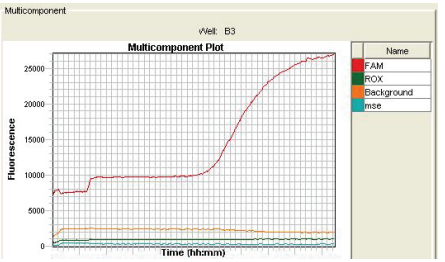
Inconsistent dye concentration. Fluctuations of ROX signal within an experiment can result in invalid gene expression data. Ensure ROX does not vary significantly within an experiment by looking at the raw background signal before the fluorescence data has been normalized.

7.8.4 When used with ROX, the TAMRA signal is diminished

TAMRA has an emission wavelength close to the absorption wavelength of ROX. Therefore, when probes labeled with TAMRA are used in reactions with master mixes that contain ROX, the signal from TAMRA can be greatly diminished. This can affect the normalized value attributed to ROX and consequently the C_q values reported. Resolve this by using another dye with an emission spectrum that does not overlap the ROX absorbance range.

10 nM ROX (too low)

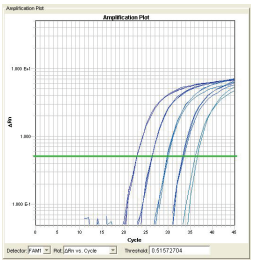


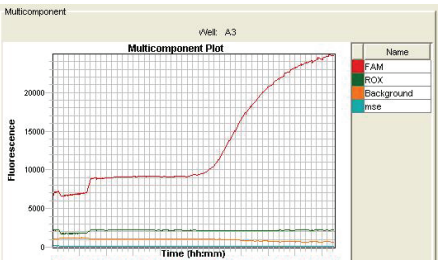


Panel A. Amplification Curves at Low ROX Concentration.

Panel B. Multicomponent Plot of Assay Dyes.

50 nM ROX (correct concentration)

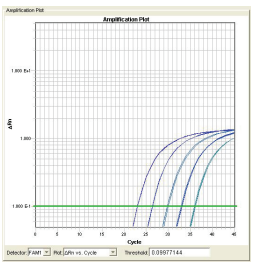


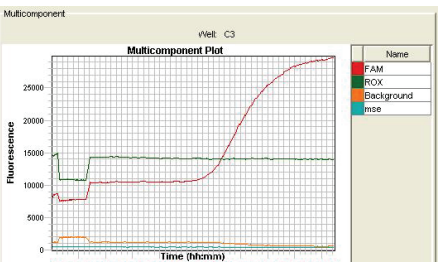


Panel C. Amplification Curves at Correct ROX Concentration.

Panel D. Multicomponent Plot of Assay Dyes.

100 nM ROX (too high)





Panel E. Amplification Curves at High ROX Concentration.

Panel F. Multicomponent Plot of Assay Dyes.

Figure 23. Using the Correct ROX Concentration. ROX concentrations were tested using Immolase DNA Polymerase (Bioline), 250 nM Probe, 500 nM primers (Assay NM_004530.2), 0.8 mM dNTP, and 3 mM $MgCl_2$. 10X serial dilutions of cDNA from Human Universal Reference RNA (20–0.002 ng) were amplified 10 min 95°C; 45 x (15 sec 95°C; 1 min 60°C). (**C** & **D**) 50 nM ROX is the optimum concentration for this assay, while (**A** & **B**) 10 nM is too low (ΔRn will be high and noisy) and (**E** & **F**) 100 nM is too high (ΔRn will be low).

7.9 Multiplexing Problems

Inability to detect expression of a target in the multiplex reaction that could be detected when analyzed individually. Increasing the number of genes to be analyzed in a single qPCR requires increasing the concentration of several of the PCR components, including $MgCl_2$ and dNTPs. Using a master mix specifically designed for multiplex PCR is recommended.

Make sure all of the primers and probes have similar melting temperatures. The melting temperatures of the probes should be 6–10°C higher than those of the primers.

Also evaluate the cross-reactivity of each assay component to ensure there is no interaction between primers and probes. The OligoAnalyzer Tool is ideal for this purpose. See Section 8.2.2 for more information.

For more information on setting up a multiplex assay, see Section 4.2.4.

C_q values for the targets in the multiplex reaction look different from those for the targets when they are analyzed separately. The C_q values should be similar whether a target is tested in a single reaction or a multiplex reaction. Limit the primers for the highest expressing targets to a 1-to-1 primer-to-probe ratio. Use double the amounts of dNTPs and enzyme in the master mix. The Mg^{2+} concentration may also need to be adjusted. See Section 4.2.4 for more information on setting up a multiplex assay.

Inability to detect the target with the least expression. A more abundant target may amplify more efficiently than a less abundant target and compromise the entire reaction. This is of particular concern in later cycles when the dNTPs and Taq polymerase are limiting. Limit the primers for the highest expressing targets to a 1-to-1 primer-to-probe ratio while increasing the primer-to-probe ratio of the other targets if necessary. Also, use a FAM-labeled probe for the target with the least expression. FAM is the brightest emitting dye and will ensure maximum sensitivity. Use double the amounts of dNTPs and enzyme in the master mix. See Section 4.2.4 for more information on setting up multiplex assays.

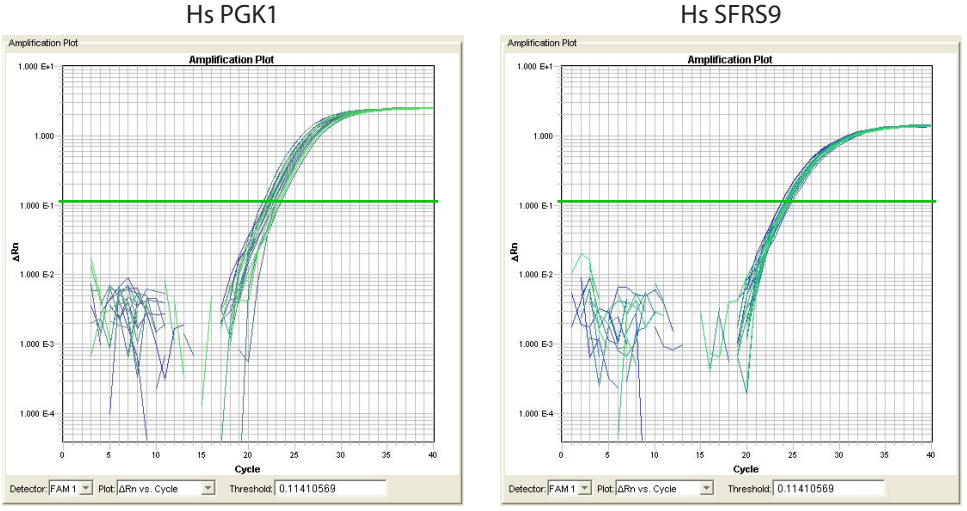
7.10 Other Observations

7.10.1 Rising Baseline

Make sure the baseline is set correctly (refer to sections 6.2 and 7.2.4). If necessary, set the baseline manually. It is also possible that there is primer–probe interaction or the primers are forming primer-dimers. Make sure to run a no template control. Evaluate the cross reactivity of each assay component. The OligoAnalyzer Tool is ideal for this purpose. See Section 8.2.2 for more information.

7.10.2 Variations in C_q of normalizer gene

The data gathered from normalization will only be as good as the control used (Figure 24). Make sure that the control has been verified as appropriate for your sample before you use it as a normalizer. The expression level of the reference gene should be the same across all conditions. See Section 6.4.2a for more information on normalization.



A. Hs PGK1.

B. Hs SFRS9.

Figure 24. Choosing the Most Stable Normalizer. RNA was isolated and cDNA generated from 18 wells of HepG2 cells transfected with control DsiRNA. Assays targeting PGK1 or SFRS9 were performed. The SFRS9 assay curves showed a smaller degree of variance compared to the PGK1 assay curves.

8. RT-qPCR Additional Resources

8.1 MIQE Publications

The information in this guide is designed to follow the standards outlined in the MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments [1]. The purpose of the MIQE guidelines is to provide a minimum set of requirements for conducting and analyzing RT-qPCR experiments, and to enable other researchers to replicate findings. It is recommended that investigators using RT-qPCR for their own experiments review those guidelines together with this guide, as it will aid in assay performance, reproducibility, and publication of experimental data.

Two updated publications, *MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments* and *Primer Sequence Disclosure: A Clarification of the MIQE Guidelines*, provide additional guidance on how to implement the MIQE standards in practice, with additional information on sample handing, experimental conditions, normalization to reference genes, and data analysis [1].

8.2 IDT Resources in Print and Online

8.2.1 SciTools® PCR Assay Design Tools

IDT offers a variety of free design and analysis tools in the online SciTools suite. For RT-qPCR designs using 5′ nuclease assays, the suite contains tools for selecting PrimeTime Predesigned qPCR Assays, and designing PrimeTime Custom qPCR Assays. In addition, the SciTools suite of analysis tools contains convenient dilution calculators, and tools for analyzing oligonucleotide properties. The SciTools software described in this section are all freely available at www.idtdna.com under the SciTools tab. There is also an excellent webinar (see Section 8.2.3) under the Support tab that demonstrates how to use these SciTools programs.

8.2.1a Predesigned qPCR Assays Tool

The IDT Predesigned qPCR Assays tool is a dedicated design tool for the PrimeTime Predesigned qPCR Assays. If your target is a human, mouse, or rat sequence, this program offers the highest level of bioinformatics analysis, including avoidance of known SNPs, BLAST search to avoid cross reaction and off-target amplification (see Section 8.3.1), and recognition of splice variants. The program is regularly updated with current sequence information from NCBI, ensuring assays are designed with up-to-date bioinformatics.

8.2.1b RealTime PCR Assay Tool

The IDT RealTime PCR tool provides effective qPCR assay design through a user-friendly interface for targets other than human, mouse, and rat, and is the recommended tool for designing custom assays. The program is customizable at many levels, and can be used to design oligonucleotides for qPCR assays with or without probes. This is useful if you plan to run qPCR assays using SYBR. The tool also includes the ability to direct the assay to specific regions of target such as select exon junctions or a particular exon found in a splice variant. qPCR conditions can be specified within the program, as can primer, probe, and amplicon parameters. If no customization is needed, this program can quickly access current NCBI data and recommend an assay for your qPCR target.

8.2.1c PrimerQuest℠ Design Tool

The PrimerQuest design tool is not a dedicated qPCR assay design program. However, it is highly customizable and useful for the design of qPCR assays with non-standard requirements. For example, you can use this design tool to direct the assay towards specific regions of your target, or you can specify primer or probe sequences. So if your design requires more demanding customization, this highly flexible program can be of great use. IDT Technical Support is available to offer assistance with this program to help you meet your specific design challenges.

8.2.2 Additional SciTools Software

In addition to assay design tools, the list of available SciTools programs includes calculators and oligonucleotide analysis tools that may be helpful for setting up RT-qPCR experiments, especially when working with custom assays.

The OligoAnalyzer® tool is the most heavily used of the IDT SciTools programs. The purpose of the OligoAnalyzer tool is to analyze the properties of input oligonucleotide sequences. It provides information about secondary structures such as hairpin and primer-dimer formation, as well as T_m, GC content, effects of modifications or buffer conditions on those properties, and an assortment of other useful information that can affect RT-qPCR or other application performance.

The DilutionCalc tool is an easy-to-use calculator designed to compute the volume of concentrated oligonucleotide stock required in order to achieve a desired dilution volume and concentration. Similarly, the ResuspensionCalc tool calculates the volume of buffer or water to add to a dry or lyophilized oligonucleotide to reach a desired final concentration.

8.2.3 Webinars

IDT produces informational webinars to guide researchers through RT-qPCR experimental design and setup. Previous webinars are archived at www.idtdna.com under the Support tab and can also be found on the IDT YouTube channel at www.youtube.com/idtdnabio.

The webinar, *Technical Tips for qPCR Assay Design and Setup*, focuses on 5' nuclease assay design and experimental setup, and provides guidance on design parameters such as T_m, GC content, amplicon size, and location of primers and probe.

Another useful webinar, *Using Free Online Tools for Oligonucleotide Analysis and Design*, discusses the use of the SciTools programs described in Section 8.2.1. This webinar covers the use of the SciTools programs specific for designing PCR and RT-qPCR experiments. It also discusses use of the OligoAnalyzer tool for basic oligonucleotide analysis, including sequence analysis for thermodynamic properties of dimers and hairpins.

8.2.4 DECODED Newsletter

The quarterly IDT *DECODED* newsletter is a free resource available in print, and online in HTML and PDF formats. It includes easily accessible articles that cover a variety of helpful scientific topics, including articles providing tips and information on many aspects of RT-qPCR. Past issues are available online at www.idtdna.com. Register for an electronic or print subscription at www.idtdna.com/decoded.

8.3 Other Resources

8.3.1 BLAST Analysis

NCBI's Basic Local Alignment Search Tool (BLAST) is an incredibly powerful tool that can be used to efficiently query the massive Genbank database to find regions of local similarity between sequences. It calculates the statistical significance of matches and can be used to select primers and probe sequences for qPCR assays. However, due to the heuristic nature of BLAST and removal of low complexity data, queries for such short sequences often return incomplete data. See the *DECODED* 1.1, April 2011 newsletter article, *Tips for Using BLAST to Locate PCR Primers*, for recommendations on how to use this tool.

8.3.2 RT-qPCR Data Analysis

The use of dedicated software, such as the qbase^{PLUS} software from Biogazelle (available at www.qbaseplus.com), for the analysis of your RT-qPCR data, can speed up data analysis, minimize errors created by manually entering data and formulas, and simplify reporting of data analysis methods in accordance with MIQE guidelines [1].

The qbase^{PLUS} software is an RT-qPCR analysis package that meets the MIQE guidelines. The software allows direct import of C_q values (data tables) from qPCR instruments from a variety of manufacturers, and provides algorithms for removal of data errors, normalization of data to one or more reference genes, and correction of inter-run variation using inter-run calibrators. The qbase^{PLUS} software also offers statistical tools for RT-qPCR data analysis, and tools for graphical presentation of analyzed data.

In addition to the qbase^{PLUS} software, Biogazelle offers a variety of other services to help investigators with design and implementation of RT-qPCR experiments, as well as educational materials and courses. For a complete overview of Biogazelle wet lab and data mining services, see www.biogazelle.com.

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11. Index

R

S

T

U

V

Z