Real-time qPCR guide: Part 3—troubleshooting

First edition

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REAL-TIME qPCR GUIDE: PART 3—TROUBLESHOOTING

First edition

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INTRODUCTION

Quantitative PCR (qPCR) assays provide a sensitive, accurate, and reproducible measure of gene expression. Unlike end-point analyses, qPCR assays quantify one or more genes of interest during amplification either using a 5′ nuclease probe or intercalating fluorescent dye protocols. Since qPCR is a complex process, many issues can influence the results.

Proper design of qPCR assays is the first step to prevent potential unexpected results. Well-designed experiments include thorough investigations of the transcript variants, exon organization, and any related genomic sequences that could potentially cross-react with the probe and primer sequences. In addition, thorough understanding of all known single nucleotide variants (SNVs) is essential to avoid primer or probe binding issues. For more information and tips to design a good qPCR assay, see IDT's Real-time qPCR guide: Part 1—assay design.

In addition to the design, ensuring accurate assay validation and data analysis will help ensure the final qPCR curves provide reliable quantification. Making sure the qPCR thermal cycler is set up appropriately for the assay, carefully determining specificity, calculating reaction efficiency, and setting up standard curves are essential steps in assay validation. In addition, improper data analysis, whether the assay is evaluated with absolute or relative quantification, can potentially create inaccurate experimental conclusions. For a thorough understanding of these parameters, consult IDT's Real-time qPCR guide: Part 2—assay validation and data analysis.

When the final qPCR amplification curve does not appear as expected, finding a quick solution is essential. This guide lists recommendations and tips from our experienced scientists that will help elucidate potential solutions for the aberrant curves. Using this guide will help you quickly determine what may have been the cause and therefore, prevent the repeating of experiments that waste valuable time, resources, and precious samples.

Let's begin.

1. TROUBLESHOOTING YOUR qPCR ASSAY DATA

As qPCR is a complex, multifaceted process, amplification curves can vary from the expected (Figure 1). 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 in the subsequent figures. Match your data with one of these and refer to the indicated section to learn what causes such curves and how to remedy them.

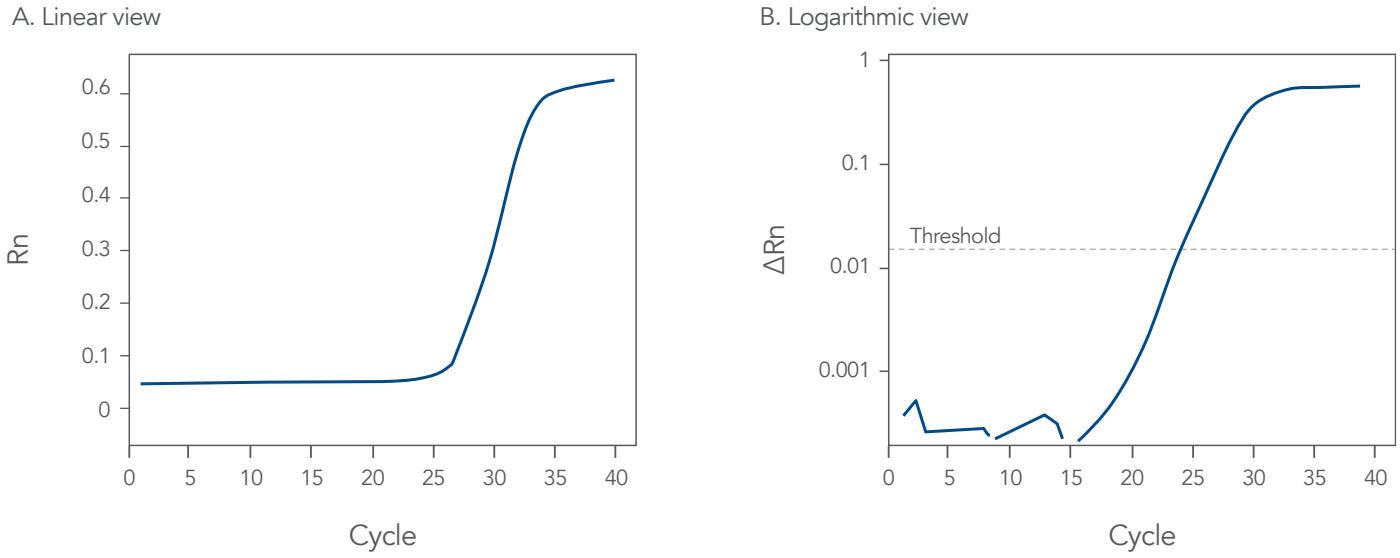

Figure 1. qPCR curves demonstrating ideal results. (**A**) The linear scale view for fluorescence (Rn) x cycle number. (**B**) Log scale view of ΔRn (shown on a $\log_{10}$ scale) x cycle number. Well defined curves include a baseline level of fluorescence for the first approximately 12–15 cycles, exponentially increasing fluorescence signal that surpasses the background.

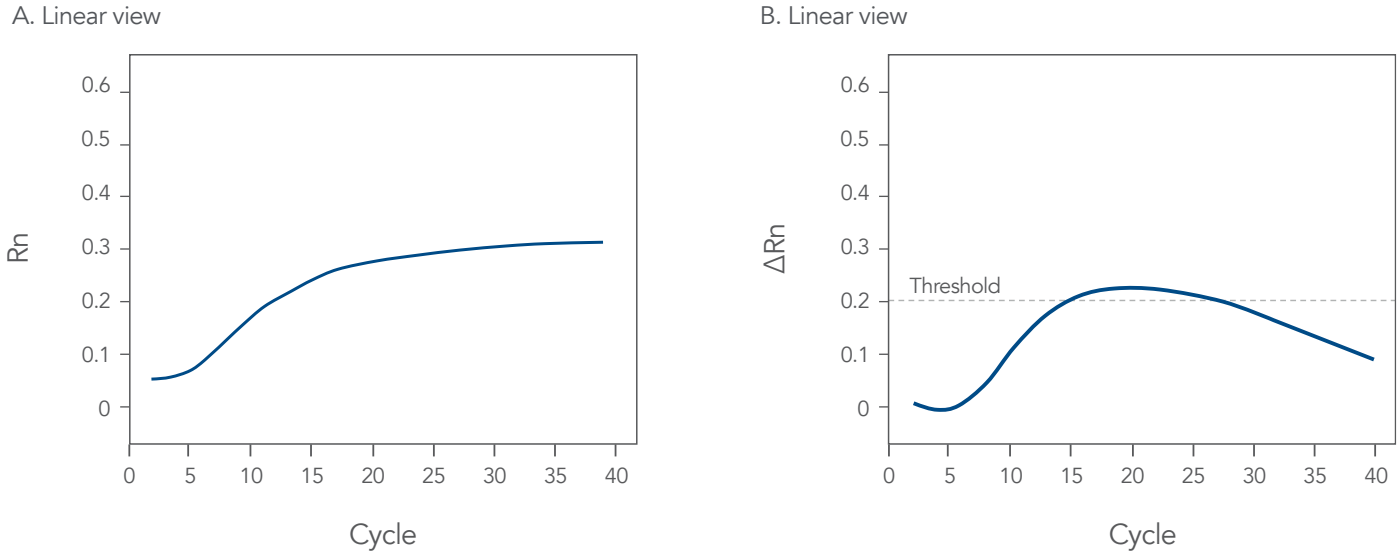

Figure 2. Too much template. (**A**) When the reaction contains too much template, the fluorescent signal will be visible in early cycles. (**B**) Since the baseline is based on the early cycle fluorescent values, the ΔRn signal will barely surpass the threshold since the baseline was wrong.

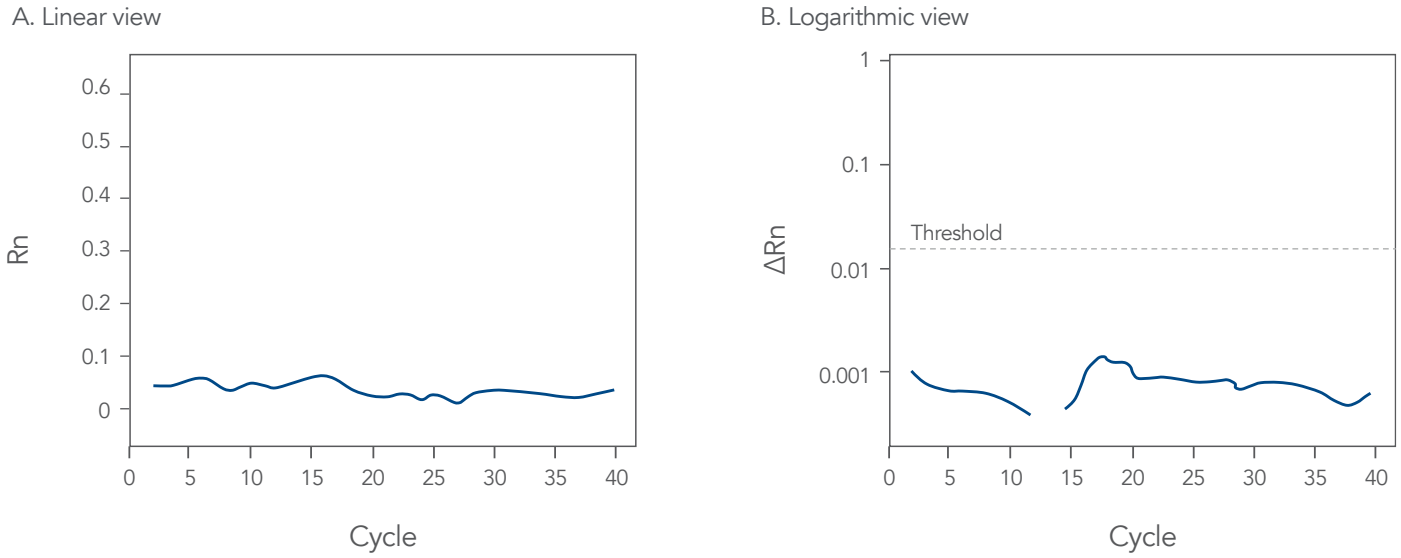

Figure 3. No amplification curve. (**A**) and (**B**) No amplification in any cycle results in no exponential curve in either the linear or log views. Several factors contribute to no signal such as no template or target in sample, missing reaction component, sample degradation, incorrect dye detector assignment, or poor assay design. (See section 1.1 for more details)

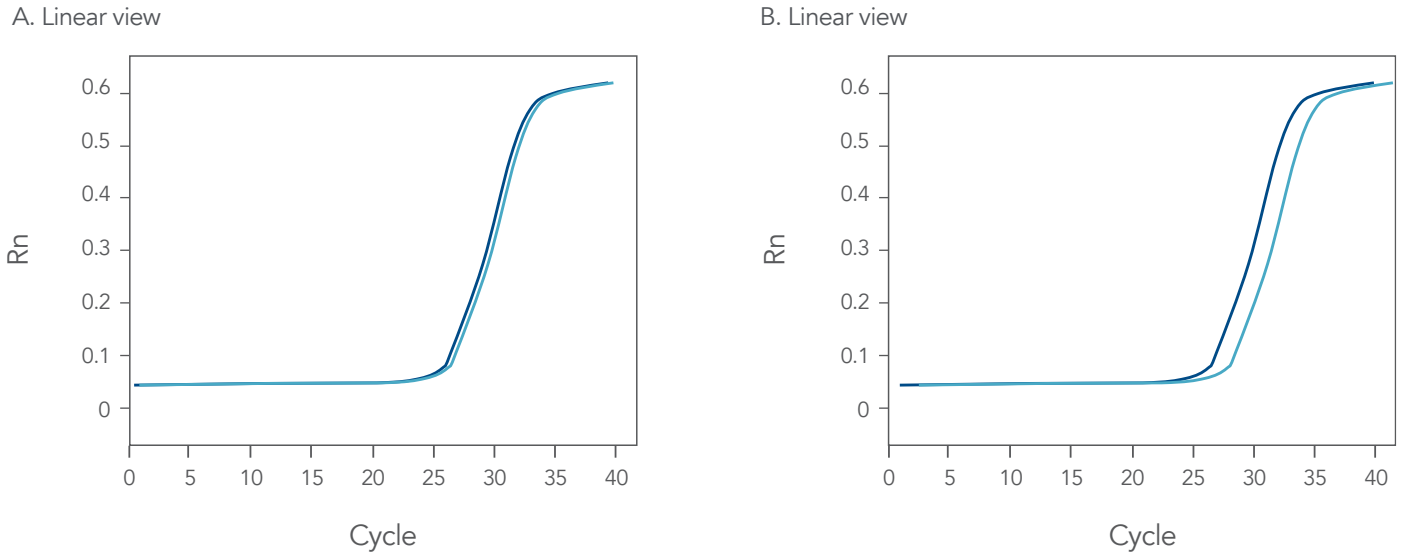

Figure 4. Inconsistent replicates. (**A**) Replicates should produce almost identical fluorescence signals. (**B**) Several factors can create inconsistent replicates, such as pipetting errors, thermal calibration of qPCR instrument, low target copy number, or inappropriate cycling conditions. (See section 1.6 for more details).

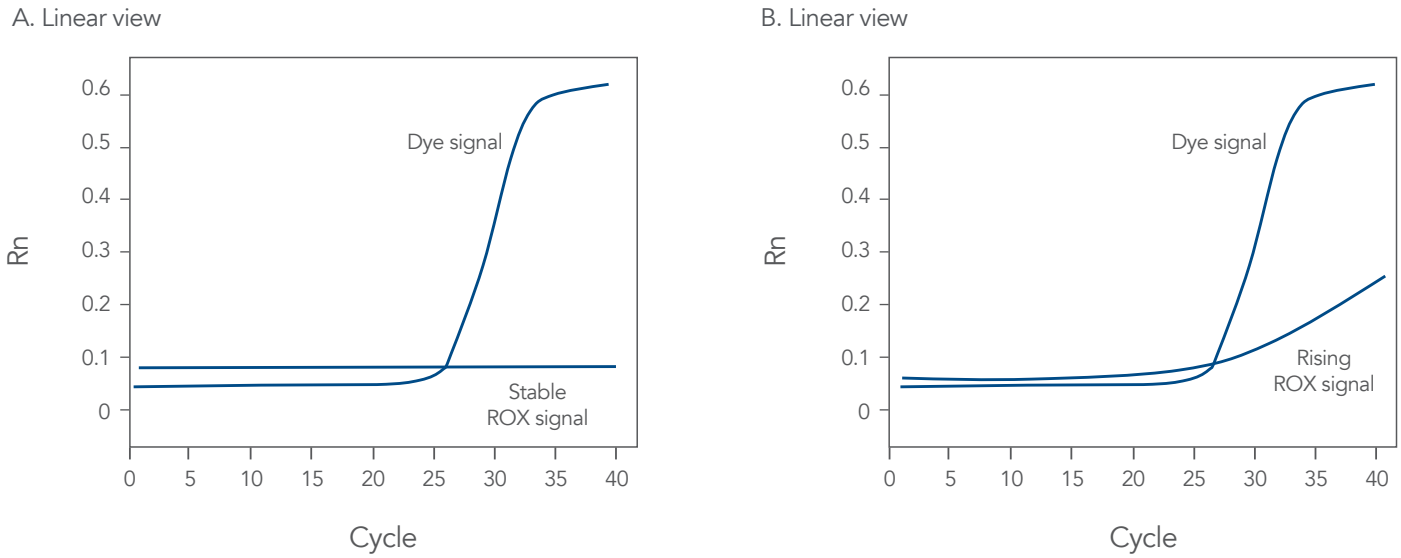

Figure 5. Rising ROX levels. (**A**) ROX fluorescence signal should be approximately the same for each cycle. (**B**) Rising ROX signals can be due to sample evaporation. (See section 1.8.1 and section 1.8.3 for more details)

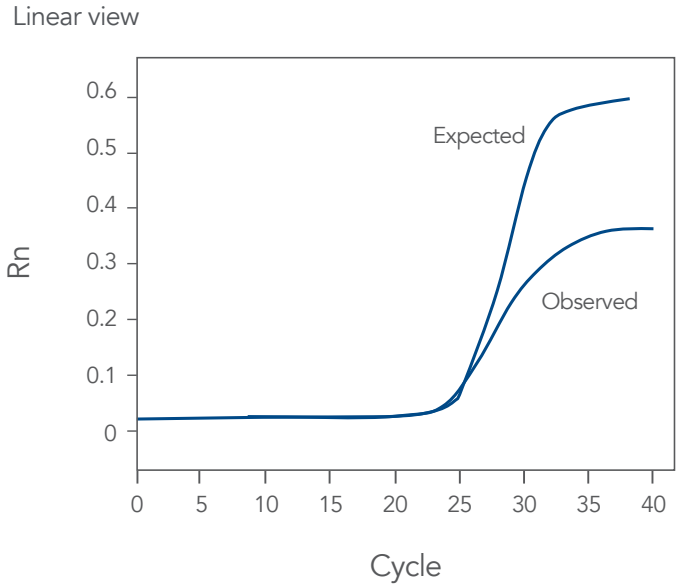

Figure 6. Low height of amplification curve. The observed fluorescence may be impeded by dye quenching by proximal G bases or differences in master mix (see sections 2 and 4 of Real-time qPCR guide: Part 1—assay design); differences in probe concentration (see this guide, section 1.2.2); inherent differences in fluorescence intensity of different dyes (see this guide, section 1.2.5); fluorophore signal overlap (see this guide, section 1.4.1); or, too much ROX in the sample (see this guide, section 1.8.1).

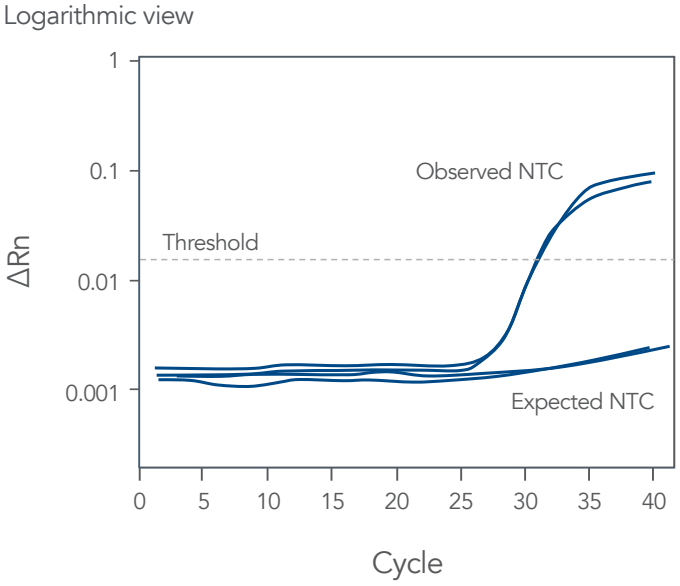

Figure 7. Amplification in the no-template control (NTC). The NTC sample should not have a fluorescent signal above threshold. When this occurs, ensure there is no template contamination in one of your reagents, or check the settings of your qPCR instrument to see if it is calibrated properly, and/or double-check that the primers are not amplifying artifacts such as primer-dimers. (See Real-time qPCR guide: Part 1—assay design, section 3b for more information.)

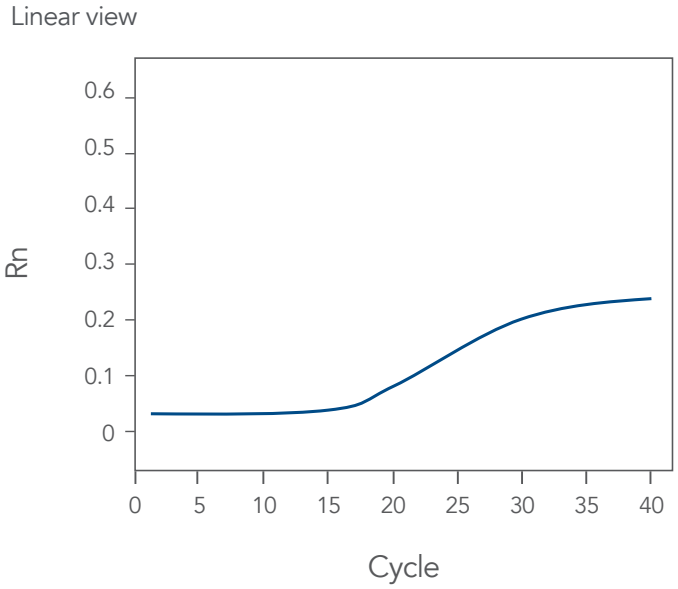

Figure 8. Poor efficiency. Poor primer design, low fluorescent dye intensity, poor instrument optics, sample inhibition, or incorrect primer concentration can lead to inefficient amplification of the target. (See section 1.3 for more details.)

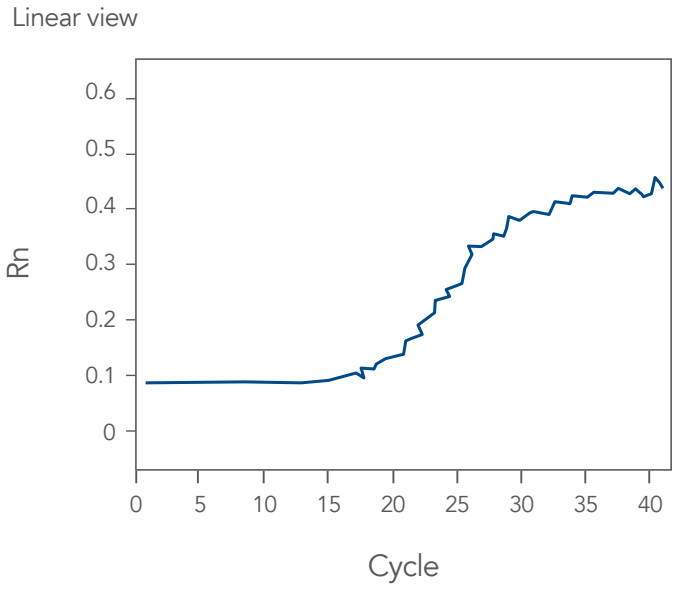

Figure 9. Noisy data. When a reaction has too much probe or insufficient reference dye, then the data can appear noisy on the linear view. (See section 1.5 for more details)

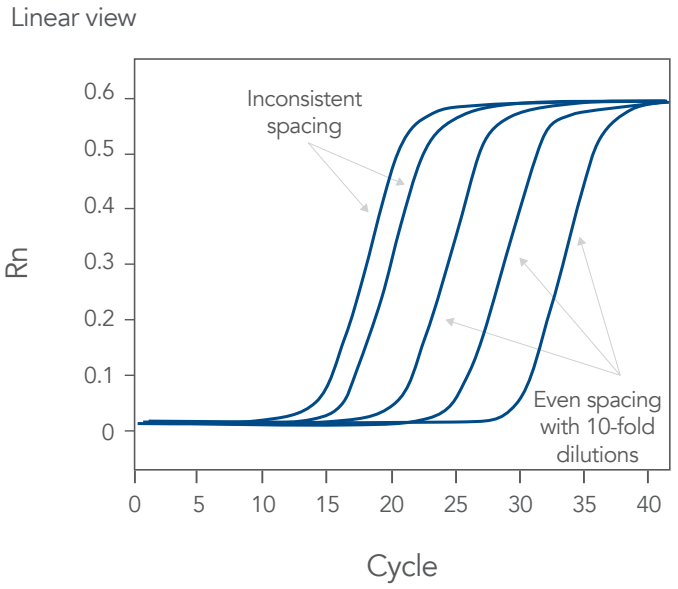

Figure 10. Inconsistent spacing for the standard curve. Careful 10-fold dilutions are necessary for evenly spaced standard curves. (See section 1.6.4 for more details)

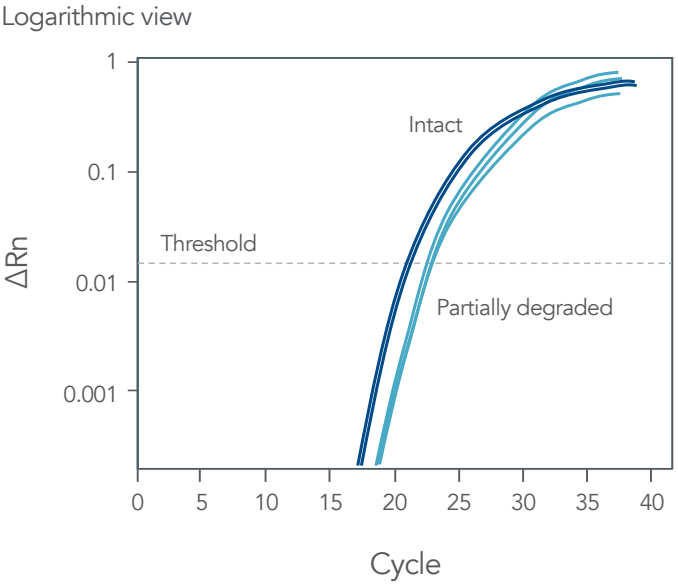

Figure 11. Later than expected C_q values. Partial degradation of the nucleic acid templates or pipetting errors can result in inconsistent replicates. (See sections 1.6.1 and 1.6.4 for more details)

1.1 Little to no amplification

1.1.1 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.

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. Note that incorrect filter settings will affect all reactions on the experimental plate that are using the same fluorophore.

1.1.2 Reaction parameters

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

Nonoptimal reverse transcription (RT) 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:

Denaturation time: Denaturation steps that are too short can result in little or no amplification since the primers and probe will be unable to access their binding sites. The initial denaturation step also inactivates any contaminating nucleases or proteases. Follow recommended guidelines as to length of denaturation for your specific DNA polymerase. Some hot-start DNA polymerases may also require a separate activation step. If short annealing/extension is desired, ensure your master mix is formulated for these conditions. When given a range of initial denaturation, choose longer times if the template DNA has greater than 65% GC content, the sample has more complexity (e.g. genomic DNA), or if the buffer has a higher salt concentration.

Annealing and extension times: Figure 12 shows the potential 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 for the master mix, 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.

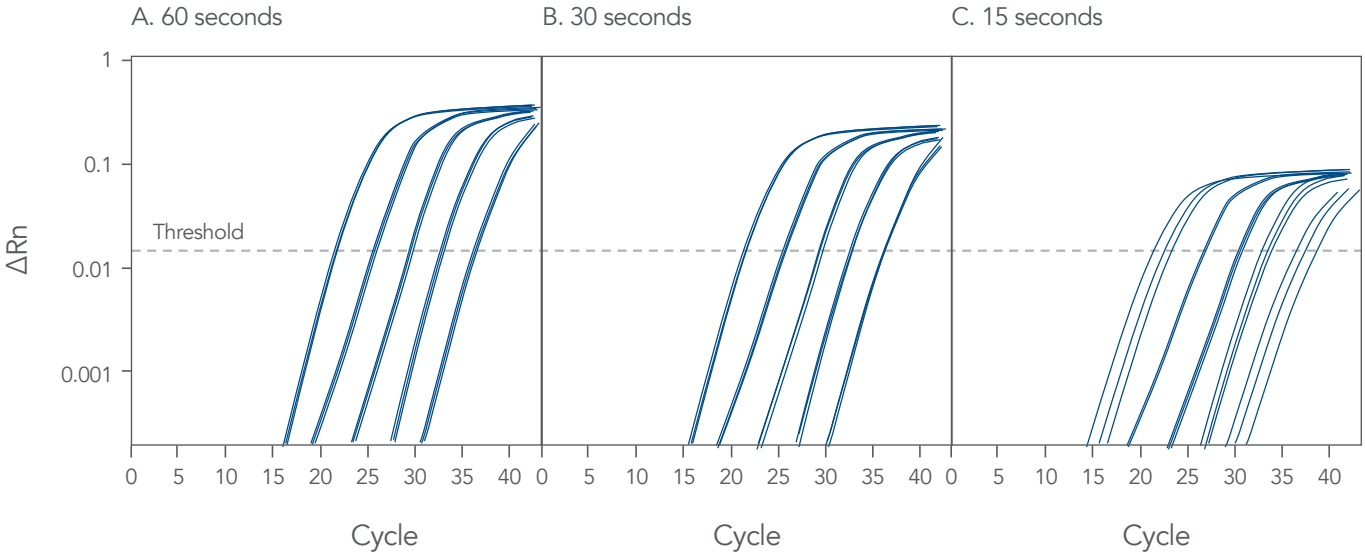

Figure 12. Potential assay results when run at different cycling conditions. (**A**) Using a recommended cycle time produces strong fluorescence that has appropriate C_q values. (**B**) and (**C**) Shortening the anneal/extend steps reduces the amount of fluorescence and creates inconsistent replicates. Ensuring that the master mix is formulated for fast cycling times will provide consistent replicates and more fluorescence even at 15-second anneal/extend times. IDT provides a versatile, high quality gene expression master mix that can be used with fast or standard cycling times, as well as singleplex or multiplex reactions. See section 2.3 for more details.

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 to optimize the assay.

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.

1.1.3 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.

Probe degraded or degrading during reaction. See section 1.7, High or variable background.

Nuclease contamination causing degradation of nucleic acids. Use a reagent such as the IDT RNaseAlert® RNase detection (Life Technologies, Inc.) or DNaseAlert Kit™ DNase detection (IDT) 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.

1.1.4 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. 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 Fragments or an Ultramer™ Oligonucleotide from IDT and cloned into a plasmid of choice. Alternatively, IDT offers Custom Gene Synthesis where the positive control sequence is already cloned in a vector. Isolating and linearizing plasmid from a single clone is the best choice for quantitation.

When the reaction has been optimized and the LOD is known, try amplifying the cDNA/ DNA sample 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 reverse transcriptase (RT) primer in the first-strand cDNA synthesis can 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 Real-time PCR guide: Part 1—assay design, section 2a for more information.

IDT related products

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 RNase or DNases. See section 2.4.3 for more information.

1.2 Low or delayed signal

1.2.1 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. Run a gel to see if the correct size of product is being amplified. Perform a BLAST search to confirm the specificity of the target and assay sequences. It is important to check that your primer and probe do not span a SNV site that can lower the binding efficiency. See section 2.5.4 for more information about NCBI's BLAST tool. IDT predesigned PrimeTime™ qPCR Probe Assays guarantee 90–110% efficiency over 4 or more orders of magnitude.

* BLAST is a registered trademark of the National Library of Medicine.

1.2.2 Reaction setup

Primers or probe were not completely resuspended. See Figure 13 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. Review the PrimeTime qPCR Assay resuspension or PrimeTime qPCR primers resuspension protocols for advice, 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 are not optimal for your sample. This can be tested by adjusting the concentration of primers and probe in 25 nM increments.

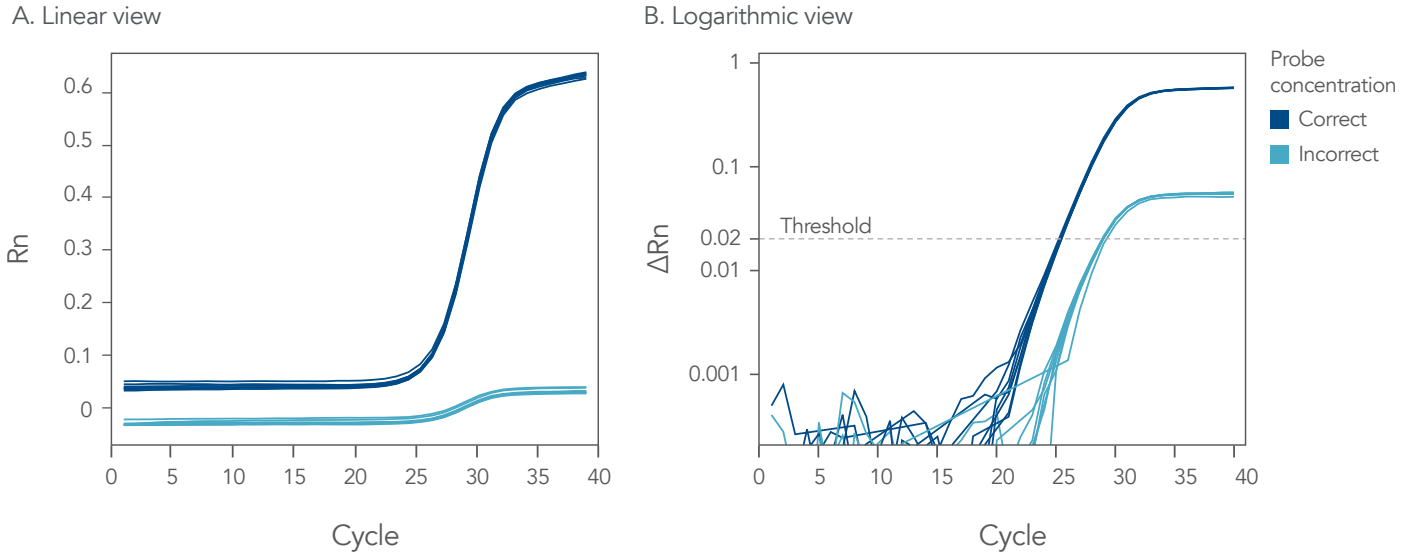

Figure 13. Low signal due to incorrect probe concentration. (**A**) Incorrect probe concentrations result in much lower fluorescence values than expected. (**B**) The low fluorescence results in ambiguous C_q values since the curve barely passes the threshold (dotted line). Reactions were run in 384-well plates using IDT Gene Expression Master Mix and 5000 copies of DNA Ultramer primers were used as input template. Each 10 µl reaction contained 400 nM of each primer, and either 200 nM of probe (correct) or 20 nM of probe (incorrect).

1.2.3 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. For more information, see the DECODED article, Sample preparation for successful qPCR.

1.2.4 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 14). 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 14, setting the baseline incorrectly can result in either delayed C_q or sloping traces that also have a delayed C_q value. See Real-time PCR guide: Part 2—assay validation and data analysis, section 4.2, for more information on setting the baseline.

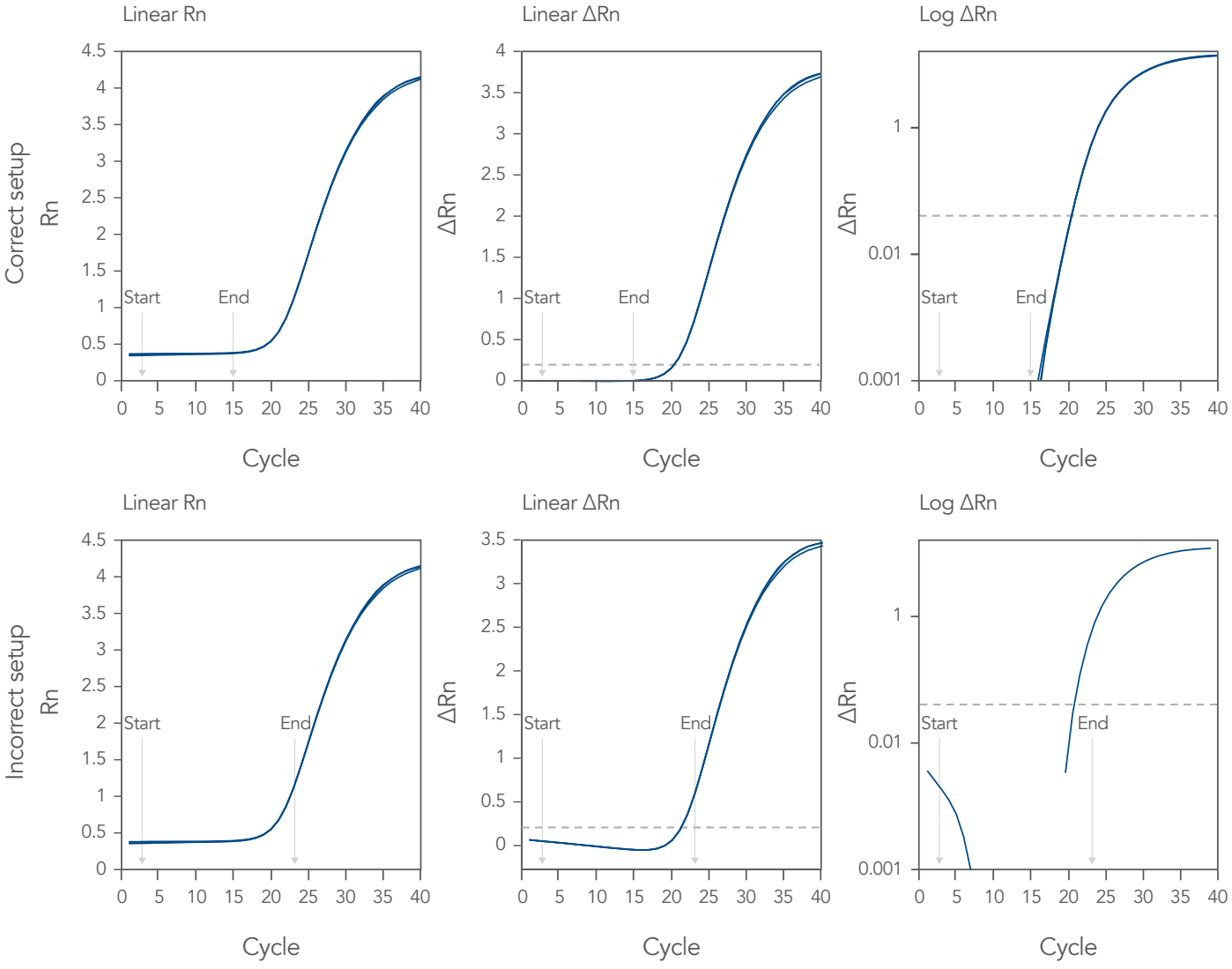

Figure 14. Correct and incorrect setup of the baseline. (Top row) Baseline start and stop values in the top three graphs are set correctly. The start and stop position of setting the baseline is clearly before the exponential phase of the fluorescence signal. (Bottom row) The baseline stop was set with the end position extending into the exponential part of the curve. Incorrect baseline settings result in either delayed C_q values or sloping traces that also have a delayed C_q value.

1.2.5 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.

1.3 Poor efficiency

1.3.1 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. Run a gel to see if the correct size of product is being amplified. Perform a BLAST search to confirm the specificity of the target and assay sequences. It is important to check that your primer and probe do not span a SNV site that can lower the binding efficiency. See Real-time qPCR guide: Part 1—assay design, section 2 for more information about NCBI's BLAST tool. IDT's predesigned PrimeTime qPCR Probe Assays guarantee 90–110% efficiency over four or more orders of magnitude.

1.3.2 Reaction setup

Efficiency of the reaction has decreased over time. Rerun an assay that has worked previously to see if the problem is specific to the assay or a general issue with another reagent. If another assay performs as expected, your assay may have degraded. 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 improvements.

1.3.3 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. Also, make sure the fluorescence is being collected at the extension step.

1.4 Excessive or unexpected signal

1.4.1 Instrument calibration

Signal detected in wrong channels. There may be overlapping fluorescent dye signals, therefore, one channel could be recording both dye emissions. 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 Cy® 5 dye (GE Healthcare, Inc.) labeled probe and look at the other dye channels to see whether there is detectable Cy® 5 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.

1.4.2 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.

1.4.3 Contamination

Reagent contamination. It is possible that the master mix or other reagents have been inadvertently contaminated with the amplicon. See section 2.2.3 for additional ways to prevent contamination.

Genomic contamination. Amplification occurring in the no reverse transcription 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 the DECODED online article, Could your PCR be affected by contamination?). When possible, design primers, probes, or amplicons to span an exon–exon junction to avoid amplification of genomic DNA (see Real-time qPCR guide: Part 1—assay design, section 2a).

1.4.4 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 >15.

1.5 Noisy data

Addition of too much probe. High probe concentration is usually due to errors in dilution or reaction setup. Verify that probe was added at the appropriate concentration. 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 as the instrument tries to normalize data using a weak, inconsistent ROX signal. See section 1.8.2 for more details.

1.6 Inconsistent replicates

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

1.6.1 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 Real-time qPCR guide: Part 1—assay design, section 4 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.

1.6.2 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.

1.6.3 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.

1.6.4 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 15 shows how degraded template can affect qPCR amplification. Poor template quality can be due to various 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. Use TE buffer (e.g., IDTE; see section 2.4.4) to resuspend the RNA sample. Check the reverse transcriptase (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.

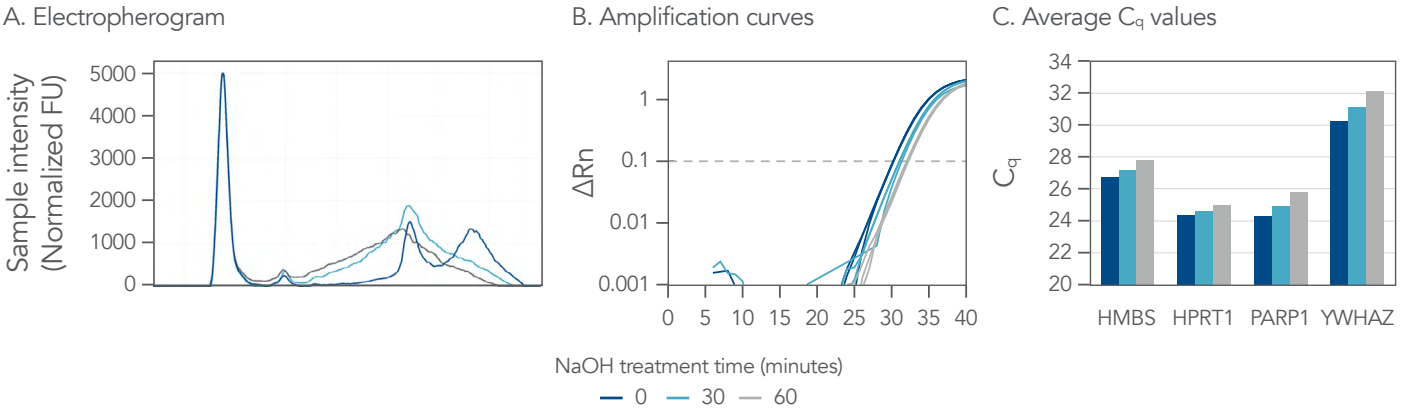

Figure 15. Poor sample quality delays C_q. To demonstrate the effect of poor RNA quality on C_q values, a 10 ng sample of Universal Human Reference RNA was left untreated (intact RNA) or treated with 10 mM NaOH for 30 min or 60 min to create increasing amounts of degradation. (**A**) The electropherogram shows the widening peak due to increased fragmentation of the RNA. (**B**) and (**C**) Amplification of degraded RNA samples delays the fluorescence signal and increases the C_q values.

1.7 High or variable background

1.7.1 Reaction setup

Addition of too much probe. High probe concentration is usually due to errors in dilution or reaction setup. 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.

1.7.2 Primer and probe integrity

Inadequate probe purification. High background can also result from poor purification of the probe. IDT performs HPLC to purify all probes. To ensure probe integrity and purity, we analyze each probe with capillary electrophoresis (CE) and mass spectrometry (MS) and provide you with these traces through your web account.

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.

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 Affinity Plus™ qPCR Probes that contain locked nucleic acids can help raise the T_m. Probes designed with Major Groove Binders (MGB) also increase the probe:target T_m to facilitate shorter probe designs. 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 Real-time qPCR guide: Part 1—assay design, Section 2 for proper design of probes and see Table 1 in this guide for absorbance ranges of quenchers and fluorophores.

IDT offers dual-quenched probes that contain a ZEN™ or TAO™ 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 Real-time qPCR guide: Part 1—assay design, Section 5 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.

1.7.3 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.

1.8 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.

1.8.1 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 16). 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.

1.8.2 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 16**.

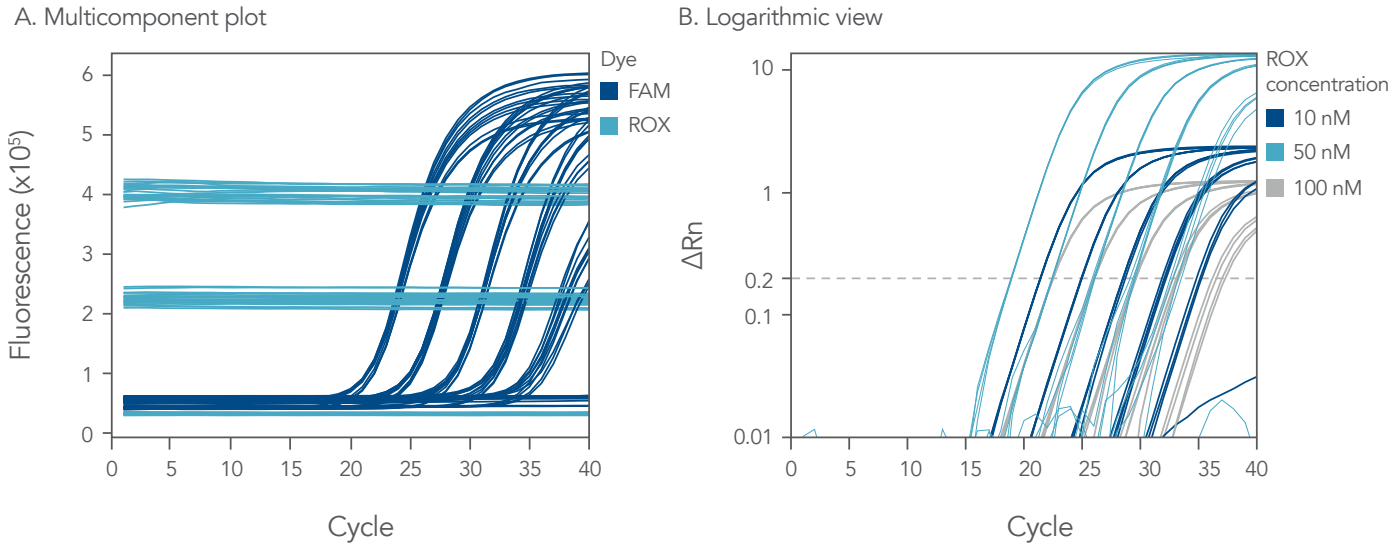

Figure 16. Using the correct ROX concentration. (**A**) ROX concentrations were adjusted from 10 nM (too low), 50 nM (normal), and 100 nM (too high). The FAM signal was the same for all three. (**B**) When the ROX signal was subtracted from the FAM fluorescence levels, high levels of ROX decreased the FAM signal. In contrast, low levels of ROX created too high of a FAM signal (note the log scale). Reactions were run in a 384-well plate using IDT Gene Expression Master Mix. Each 10 µl reaction contained a 5-log dilution of cDNA from Human Universal Reference RNA (50–0.005 ng).

1.8.3 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.

1.8.4 When used with ROX, my reporter dye signal is diminished

Some reporter dyes, such as TAMRA, have an emission wavelength close to the absorption wavelength of ROX. Therefore, when probes labeled with these dyes are used in reactions with master mixes that contain ROX, their signal 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.

1.9 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 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 2.3 for more information.

For more information on setting up a multiplex assay, see Real-time qPCR guide: Part 1—assay design, section 2.d.

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 Real-time qPCR guide: Part 1—assay design, section 2.d. 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 multiplex reaction. This is of 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 Real-time qPCR guide: Part 1—assay design, section 2.d. for more information on setting up multiplex assays.

1.10 Other observations

1.10.1 Rising baseline

Make sure the baseline is set correctly (refer to Real-time qPCR guide: Part 2—assay validation and data analysis, section 2.2 and this guide, Section 1.2.4). 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 Real-time qPCR guide: Part 1—assay design, section 6 for more information.

1.10.2 Variations in C_q of normalizer gene

The data gathered from normalization will only be as good as the control used (Figure 17). 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 Real-time qPCR guide: Part 2—assay validation and data analysis, section 4.1.2a for more information on normalization.

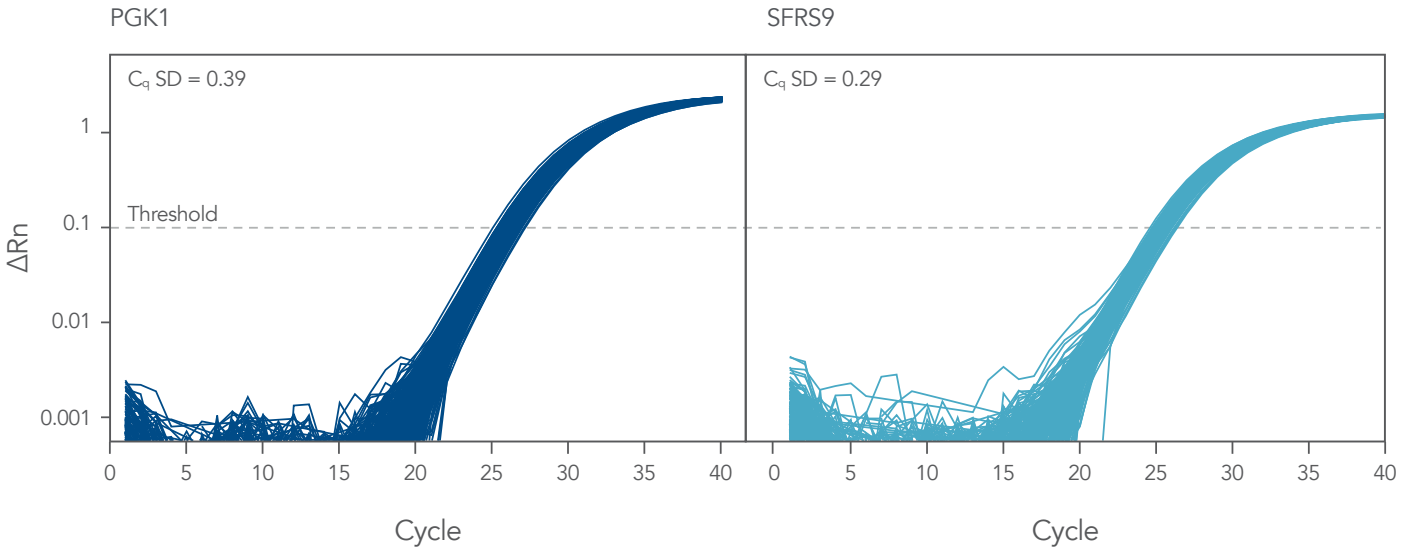

Figure 17. Choosing the most stable normalizer. RNA was isolated and cDNA generated from 18 wells of HeLa cells transfected with control DsiRNA. Assays targeting PGK1 (left) or SFRS9 (right) were performed. The SFRS9 assay curves showed a smaller degree of variance compared to the PGK1 assay curves.

2. qPCR REAGENTS, ASSOCIATED PRODUCTS, SOFTWARE TOOLS, AND ADDITIONAL RESOURCES FROM IDT

2.1 Complete assays, probes, primers, and master mix

We have developed a selection of qPCR products to assess gene expression. These include predesigned probe- and primer- based assays covering transcriptome targets in human, mouse, and rat. Custom assays may be created for any sequence from any species using the PrimerQuest™ Tool.

You can obtain IDT qPCR reagents as ready-to-use solutions with PrimeTime probe- or primer-based assays and master mix or create a completely custom option.

2.1.1 PrimeTime Predesigned qPCR Assays

PrimeTime Predesigned qPCR Assays are available for the majority of human, mouse, and rat transcripts in the NCBI database—where primer and probe designs meet our strict selection criteria. These assays are designed using a proprietary algorithm. In addition to optimized oligo T_m (base composition, oligo length, etc.), the bioinformatic calculations account for factors, such as SNPs, cross-react searches to avoid off-target amplification, recognition of splice variants, and secondary structure predictions. The IDT web-based ordering system (Real-time qPCR guide: Part 2—assay validation and data analysis, section 5) allows users to filter, sort, and select assays based on characteristics like exon location, splice variant targets, RefSeq number, or gene symbol.

It is important to note that, unlike many other commercially available inventoried assays, PrimeTime Predesigned qPCR Assays are not manufactured and stocked, but are predesigned only. In this way, updates to sequence and SNP databases can be considered.

Predesigned assays are guaranteed to perform with PCR efficiencies of 90–110% and R^2 >0.99. If you find an assay does not meet these criteria, it will be replaced with another at no additional charge. We also provide primer sequences with each order to assist with best practices in research reporting and reproducibility—specifically, sequence transparency [1].

Assays consist of a forward primer, a reverse primer, and a qPCR probe delivered in tubes or 96-well plate format. In addition, for the standard and XL sizes, dye-quencher combination and custom primer-to-probe ratio can be specified to meet unique experimental needs. Each oligonucleotide undergoes 100% QC by mass spectrometry*, and all QC results are provided free of charge to you on the IDT website.

2.1.2 PrimeTime Custom qPCR Assays

Custom 5′ nuclease assays can also be designed for any sequence in any species using the PrimerQuest Tool (see section 2.5.2). The tool includes optimized preset design parameters for PCR and qPCR or customizable parameters for other applications.

PrimeTime Custom qPCR Assays consist of a forward primer, a reverse primer, and a qPCR probe delivered in tube and 96-well plate format. In addition, for the standard and XL sizes, dye-quencher combination and custom primer-to-probe ratio can be specified to meet unique experimental needs. Each oligonucleotide undergoes 100% QC by mass spectrometry*, and all QC results are provided free of charge to you on the IDT website.

Learn more about IDT PrimeTime qPCR Probe Assays.

* With the exception of mixed base oligos, which could potentially represent multiple sequences and therefore cannot be accurately evaluated by ESI mass spectrometry.

2.1.2a Gene sets

We have compiled suggested gene lists for targeting some commonly studied pathways in human, mouse, and rat. These include housekeeping, apoptosis, cytokines, growth factor, and tumor metastasis genes. Gene lists for additional human signaling pathways (Notch, TLR, WNT, etc.), biochemical pathways, and genes that have been associated with certain cancers, are also available as an Extended Gene Set List. View these lists on the **Gene Sets for qPCR page**.

2.1.2b Reference gene assays for normalization

We provide a selection of predesigned assays for common normalization (housekeeping) genes for human, mouse, and rat. Review the details of these housekeeping gene sets on the **Gene Sets for qPCR page**.

2.2 qPCR probe types

2.2.1 Minor Groove Binder (MGB) qPCR probe assays and probes

MGB probes include a 5′ fluorescent dye and a 3′ non-fluorescent quencher (NFQ) that binds to the minor groove of the DNA and stabilizes the connection between the probe and its target. MGB probes have increased melting temperatures (T_m); therefore, probe lengths can be decreased. IDT offers MGB qPCR probes in an assortment of reporter-dye combinations in a variety of sizes.

2.2.2 ZEN and TAO Double-Quenched Probes

ZEN/Iowa Black™ FQ and TAO/Iowa Black™ RQ double-quenched probes provide superior performance compared to traditional single-quenched probes. While traditional probes have approximately 20–30 bases between the fluorophore and the quencher, the internal ZEN or TAO quencher decreases that length to only 9 bases. This shortened distance, particularly when combined with the traditional 3′ end quencher, leads to a much more thorough quenching with much lower background and enables the use of much longer probes for designing in AT-rich target regions. In addition to the significantly decreased background, double-quenched probes also provide consistently reduced C_q values and improved precision when compared to traditional probes. Use of double-quenched probes can deliver both increased sensitivity and precision for your qPCR experiments.

2.2.3 Affinity Plus probes with locked nucleic acids

Affinity Plus probes contain up to six locked nucleic acid monomers. When incorporated into a probe, locked nucleic acids impart heightened structural stability, leading to increased hybridization and melt temperature (T_m). The number of Affinity Plus monomers allow for flexible T_m adjustments in designing shorter, more specific qPCR probes. For more information see **Affinity Plus qPCR probes** and the **locked nucleic acid** technology page.

2.2.4 MGB Eclipse Probes

MGB Eclipse® Probes (ELITech Group) and GMP companion primers are provided by our GMP and ISO 13485-certified production group and are ideal for qPCR assays for a variety of applications. Several fluorophore options (FAM, HEX, TET, and Yakima Yellow® Dye (ELITech Group)) allow you to ensure compatibility with your instrument and more easily design multiplex assays.

2.2.5 Freedom Dyes

Freedom™ Dyes are license-free, fluorescent dyes, for use in a variety of applications. They are available for commonly used dye wavelengths and can be paired with ZEN, TAO, and Iowa Black quenchers to create qPCR probes with lower background and higher signal.

For more information, download the **PrimeTime Custom qPCR Probes Flyer**.

2.2.6 Table of common fluorescent dyes

Table 1. Commonly used fluorophores and quenchers

Fluorophore*	Emission wavelength (nm)	Quencher
6-FAM*	520	ZEN/Iowa Black FQ
TET*	539	
Yakima Yellow®	551	
SUN™	554	
JOE*™	555	
HEX*	555	
MAX™	557	Iowa Black RQ†
Cy® 3	564	
ATTO™ 550	575	
TAMRA	583	
ATTO™ 565	591	
ROX	608	
Texas Red® -X	617	
ATTO™ 633	653	
ATTO™ 647N	662	
Cy® 5	668	TAO/Iowa Black RQ‡

* Probes with 6-FAM, TET, HEX, or JOE fluorophores are also available as traditional, single-quenched probes with either Iowa Black FQ (license free) or Black Hole Quencher®-1 (additional third-party licenses required for diagnostic use of BHQ1).

† Black Hole Quencher-2 may also be used as a quencher (additional third-party licenses required for diagnostic use).

‡ Cy® 5 is also available as a traditional, single-quenched probe with Iowa Black RQ (license free) or Black Hole Quencher-2 (additional third-party licenses required for diagnostic use).

2.3 PrimeTime Gene Expression Master Mix

PrimeTime Gene Expression Master Mix is optimized to support probe-based qPCR assays for gene expression analysis. This master mix is guaranteed to provide assay efficiencies >90% when used with PrimeTime qPCR Assays in two-step RT-qPCR. It is also compatible with other primers and probes.

Each order includes a 2X master mix solution (antibody-mediated, hot-start DNA polymerase; dNTPs; $MgCl_2$; enhancers; and stabilizers) and a separate reference dye stock solution.

PrimeTime Gene Expression Master Mix is shipped at ambient temperature. Elimination of shipping on dry ice saves you research money, minimizes shipping delays, and benefits the environment.

To view our extensive testing that shows ambient shipping does not impact the function of the master mix, see our **white paper**.

Learn more about **PrimeTime Gene Expression Master Mix**.

2.4 Other useful reagents from IDT

2.4.1 Ultramer DNA Oligonucleotides

IDT synthesis systems and chemistries allow high fidelity synthesis of very long single-stranded oligonucleotides (up to 200 bases). Ultramer DNA Oligonucleotides are suitable for demanding applications such as cloning, shRNA, mutagenesis, and gene construction. Ultramer Oligonucleotides serve as excellent controls.

Learn more about **Ultramer DNA Oligonucleotides**.

2.4.2 gBlocks Gene Fragments

Up to 3000 bp in length, these double-stranded DNA fragments are constructed using Ultramer Oligonucleotides and are sequence-verified. gBlocks fragments serve as excellent controls. The target sequence can be ordered as a **gBlocks Gene Fragment** or an **Ultramer Oligonucleotide** from IDT and cloned into a plasmid of choice. Alternatively, IDT offers **Custom Gene Synthesis** where the positive control sequence is already cloned in a vector. Isolating and linearizing plasmid from a single clone is the best choice for quantitation.

Learn more about **gBlocks Gene Fragments** and **Custom Gene Synthesis**.

2.4.3 Nuclease detection and control reagents

We also offer several reagents for rapid detection and elimination of RNases and DNases. The detection assays, RNaseAlert Substrate and DNaseAlert, are effective for testing lab reagents, equipment, and other lab supplies. Results can be read visually for qualitative assessment of contamination or quantified using fluorometry. The IDT Nuclease Decontamination Solution that irreversibly inactivates nucleases and that can be applied to most laboratory surfaces is also available.

Learn more about **IDT nuclease detection and control reagents**.

2.4.4 IDTE Buffer

We provide 1X TE Buffer (10 mM Tris, 0.1 mM EDTA; available at pH 7.5 or 8.0) for initial resuspension and storage of DNA oligonucleotides. DNA oligonucleotides can be damaged by prolonged incubation or storage in 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.

Learn more about **IDTE Buffer and other reagents** available from IDT.

2.4.5 RNase-free H_2O

Nuclease-Free Water is convenient for quick dilutions of storage stocks, or for especially short-term storage. For more information on various storage media and their effects on oligo stability, click see the IDT DECODED online article, **Storing oligos: 7 things you should know**.

2.5 Software tools for assay design and primer evaluation

2.5.1 Predesigned qPCR Assay selection tool

The **IDT Predesigned qPCR Assay** selection tool is a dedicated design tool for the library of PrimeTime Predesigned qPCR Assays. If your target is a human, mouse, or rat sequence, this program offers the highest level of bioinformatics analysis, including BLAST search to avoid cross reaction and off-target amplification, and recognition of splice variants.

2.5.2 PrimerQuest Tool

The **PrimerQuest design tool** 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. Use the PrimeQuest Tool to adjust reaction conditions, add a probe to a set of previously designed primers, define primer positions, and include or exclude sequences from the assay designs. So, if your design requires more demanding customization, this highly flexible program can be a great resource. IDT Technical Support is available to help you use this program to meet your specific design challenges. Contact us at **applicationsupport@idtdna.com**.

2.5.3 PrimeTime Multiplex Dye Selection tool

The **PrimeTime Multiplex Dye Selection Too**l helps you select dye combinations that are compatible with your qPCR instrument. Multiplexing 2–5 qPCR targets in a single reaction can save time and money.

2.5.4 NCBI BLAST tool

NCBI's Basic Local Alignment Search Tool (BLAST tool) 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. For more information, see the IDT DECODED newsletter article, **Tips for using BLAST to locate PCR primers**.

2.5.5 Additional tools and calculators

In addition to the assay design tools described above, additional SciTools™ Web Tools include calculators and an oligonucleotide analysis tool that may be helpful for setting up RT-qPCR experiments, especially when working with custom assays. Here is a review of several tools that can prove helpful when setting up qPCR experiments.

2.5.5a OligoAnalyzer Tool

OligoAnalyzer Tool is the most popular IDT SciTools program. This tool analyzes the properties of oligonucleotide sequences. By simply inputting your sequence, you can find out its length, GC content, melting temperature range, molecular weight, extinction coefficient, and optical density. The program also provides information about secondary structures, such as hairpin and primer-dimer formation, as well as mismatches, 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.

2.5.5b Dilution Calculator

The **Dilution Calculator** is an easy-to-use calculator designed to compute the volume of concentrated oligonucleotide stock required to achieve a desired dilution volume and concentration.

2.5.5c Resuspension Calculator

The **Resuspension Calculator** determines the volume of buffer or water to add to a dry or lyophilized oligonucleotide to reach a desired final concentration.

2.5.6 RT-qPCR data analysis

The use of dedicated software, such as the qbasePLUS 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 qbasePLUS 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 qbasePLUS software also offers statistical tools for RT-qPCR data analysis, and tools for graphical presentation of analyzed data.

In addition to the qbasePLUS 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**.

2.6 Other qPCR resources from IDT

We have devoted significant research resources towards improving qPCR assay performance and reproducibility both for our customers and for our own researchers. This guide is Part 3 in a series meant to communicate our experiential expertise. Access the other 2 volumes here:

Real-time qPCR assay guide: Part 1—assays design

Real-time qPCR assay guide: Part 2—assay validation and data analysis

Contact our **Scientific Application Support** team for specific questions about your qPCR assay design, data analysis, and troubleshooting.

IDT also provides an extensive collection of educational **video tutorials & webinars** and **articles** on qPCR topics.

Visit us at **www.idtdna.com** for more information.

3. REFERENCES

1. Bustin SA, Benes V, et al. (2009) The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem, 55(4):611–622. Updates: Bustin SA, Beaulieu JF, et al. (2010) MIQE précis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol.11:74–78, and Bustin SA, Benes V, et al. (2011) Primer sequence disclosure: A clarification of the MIQE Guidelines. Clin Chem, 57:919–921.

Real-time qPCR guide: Part 3—troubleshooting

Technical support: applicationsupport@idtdna.com

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