SsoAdvanced™ Universal Probes Supermix

Instruction Manual

For use with probe-based real-time PCR applications
on all real-time PCR instruments

Catalog #172-5280
172-5281
172-5282
172-5284
172-5285

BIO-RAD

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For help and technical advice, please contact the Bio-Rad Technical Support department. In the United States, the Technical Support department is open Monday–Friday, 5:00 AM–5:00 PM, Pacific time.

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Legal Notices

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SsoAdvanced™ Universal Probes Supermix

Catalog #	Supermix Volume	Kit Size
172-5280	2 ml (2 x 1 ml vials)	200 x 20 µl reactions
172-5281	5 ml (5 x 1 ml vials)	500 x 20 µl reactions
172-5282	10 ml (10 x 1 ml vials)	1,000 x 20 µl reactions
172-5284	25 ml (5 x 5 ml vials)	2,500 x 20 µl reactions
172-5285	50 ml (10 x 5 ml vials)	5,000 x 20 µl reactions

Shipping and Storage

The SsoAdvanced universal probes supermix is shipped on dry ice. Upon receipt, the supermix should be stored at –20ºC in a constant temperature freezer and protected from light. When stored in these conditions, the supermix is guaranteed for one year. When stored at 4ºC, the supermix is guaranteed for three months. To avoid excess freeze-thaw cycles, we recommend preparing aliquots for storage.

Kit Contents

SsoAdvanced universal probes supermix is a 2x concentrated, ready-to-use reaction master mix optimized for dye-based real-time PCR on any real-time PCR instrument (ROX-independent and ROX-dependent). It contains antibody-mediated hot-start Sso7d fusion polymerase, dNTPs, MgCl2, probes, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

Instrument Compatibility

This supermix is compatible with all Bio-Rad and ROX-dependent Applied Biosystems real-time PCR instruments, and with the Roche LightCycler LC480, Qiagen Rotor-Gene Q, Eppendorf Mastercycler ep realplex, and Stratagene Mx real-time PCR systems.

Product Use Limitations

The SsoAdvanced universal probes supermix is intended for research use only, and is not intended for clinical or diagnostic use.

Technical Assistance

Bio-Rad Laboratories takes great pride in providing best-in-class technical support through our online, telephone, and field support. To obtain support, please visit www.bio-rad.com, call 1.800.4.BIORAD, or contact your local field applications scientist.

Quality Control

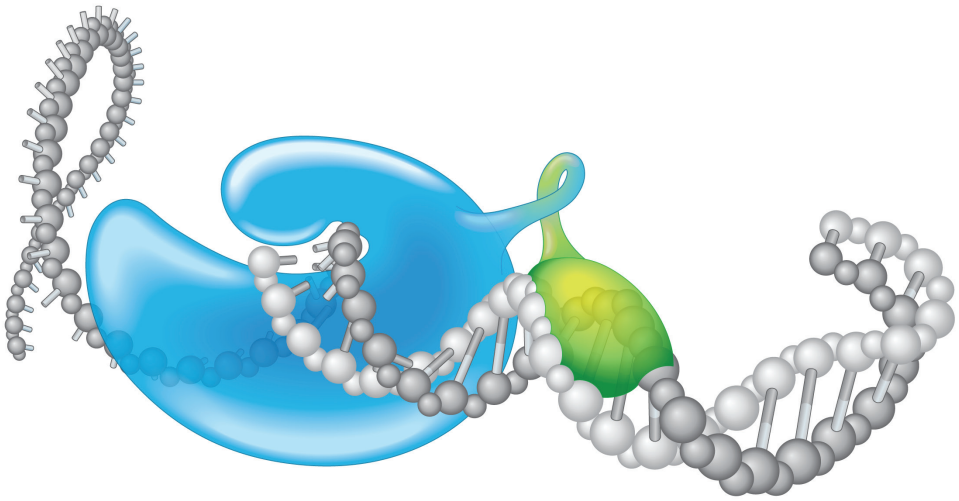
SsoAdvanced universal probes supermix demonstrates high PCR efficiency and linear resolution over a wide linear dynamic range. Stringent specifications are maintained to ensure lot-to-lot consistency. This product is free of detectable DNase and RNase activities.

Sso7d Fusion Enzyme Technology

Bio-Rad introduced our next generation of real-time PCR supermixes using our patented Sso7d fusion protein technology, delivering a reagent that provides effective performance in a wide range of qPCR applications. The dsDNA-binding protein, Sso7d, stabilizes the polymerase-template complex, increases processivity, and provides greater speed and reduced reaction times compared to conventional DNA polymerases, without affecting PCR sensitivity, efficiency, or reproducibility.

Key Features and Benefits

- **Fast qPCR results and high performance** — the Sso7d fusion polymerase and optimized buffer deliver fast reaction times via instant antibody hot-start polymerase activation and rapid polymerization kinetics to generate exceptional qPCR results in less than 30 min
- **Minimal inhibition of PCR** — the polymerase's increased resistance to PCR inhibitors ensures maximum efficiency, sensitivity, and reproducibility
- **Single copy detection** — data illustrate high sensitivity with amplification and detection from a single copy of target gene
- **Robust discrimination and reproducibility** — efficient discrimination and reliable quantification can be obtained from 1.33-fold serial dilutions of input template
- **GC-rich targets** — ability to amplify targets where other Taq-based supermixes may be challenged



Educational Resources

Understanding the Basics

To learn more about similarities and differences between PCR and real-time PCR, understand how SYBR® Green and probe-based chemistries function, and see how data are collected and interpreted, please view our interactive tutorial Understanding Real-Time PCR.

Reagent Evaluation and Comparison Tutorials

Reverse Transcription

When comparing two different reverse transcription kits, often not all characteristics of the reverse transcription (RT) reaction are tested. The end result is that a decision is made using a limited set of data and criteria. The following protocol and exercise have been written in an effort to create a more robust, reliable, and reproducible method of testing sensitivity, efficiency, and other critical characteristics when comparing reagent providers for reverse transcription kits. Reagent Comparison Guide for Real-Time PCR

To view an interactive tutorial and learn about reverse transcription chemistry, enzymes, and priming methods, as well as how to perform a reagent comparison, please click here. Understanding Reverse Transcription

Supermixes

When comparing two different supermixes, often not all characteristics of the PCR reaction are tested. The end result is that a decision is made using a limited set of data and criteria. The Reagent Comparison Guide for Real-Time PCR was written in an effort to create a more robust, reliable, and reproducible method of testing sensitivity, efficiency, and other critical assay characteristics when comparing reagent providers for use on real-time PCR systems.

To view an interactive tutorial and learn about supermix chemistry and enzymes, as well as how to perform a reagent comparison, please click here. Understanding Real-Time PCR Supermixes

Protocol

This manual is intended for use with probe-based assays on all real-time PCR systems using a broad range of cycling conditions, template and primer input concentrations, and fast or standard run times.

Sample Preparation Considerations

RNA Samples

- Isolate RNA using the appropriate method for the given sample type (Aurum™ total RNA mini kit for cell lines, Aurum total RNA fatty and fibrous tissue kit for tissue samples)
- Compare the expected yield to the actual yield to ensure the isolation method yielded the appropriate RNA concentrations (5–30 pg per cell, 0.1–4 µg per mg of tissue). When the yield is less than expected, this may lead to suboptimal qPCR data results, due to less than ideal quality samples resulting from suboptimal sample prep workflow
- When the RNA will be used for RT-qPCR, it is recommended that you treat the sample with DNase to remove residual contaminating DNA. DNase treatment is also a good idea when isolating RNA from tissues that are high in DNA, as the excess DNA may affect downstream applications
- Store the RNA in an appropriate solution
  - 0.1 mM EDTA (in DEPC-treated ultrapure water)
  - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0)
- Store the RNA at –80ºC in single-use aliquots

RNA Integrity and Purity

- Use the Experion™ automated electrophoresis system or the Agilent Bioanalyzer to evaluate the integrity of the RNA sample. When using multiple samples in the comparison, ensure that the RQI/RIN numbers are similar to ensure accurate qPCR results
- Use an agarose gel to assess RNA integrity if the above systems are not available. Apply the same analysis concepts. High quality RNA will yield two clean peaks, 18s and 28s. Degraded RNA will appear as a smear on the gel
- To assess purity, evaluate the following spectrophotometer readings:
  - A260/A280 >2.0 for pure RNA
  - A260/A230 ~2.0 for pure RNA
    - Lower ratios are indicative of contaminants from salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate

DNA Samples

- Isolate DNA using the appropriate method for the given sample type (for example, column purification for cell lines, phenol/chloroform or column purification for tissue samples)
- Store the DNA in an appropriate solution
  - 0.1 mM EDTA (in DEPC-treated ultrapure water)
  - TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0)
- Store the DNA at –80ºC in single-use aliquots
- Assess DNA quality with an agarose gel; a single band indicates high integrity DNA, whereas a smear indicates degraded DNA
- Assess the DNA purity using a spectrophotometer for the following:
  - A260/A230 >1.5 (lower ratios may be attributed to carryover guanidine, and/or inhibitors like humic acid and organics)
  - A260/A280 1.7–2.0 (lower ratios are indicative of contaminants from salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate)
  - Higher ratios may be indicative of RNA contamination

Tips:

- Heat treating DNA may be required prior to qPCR to relax strong secondary structure
- Using a restriction digest enzyme may be required for select qPCR applications, such as copy number variation, to reduce signal-to-noise ratio.

Plasmid Samples

- Prepare plasmids using an appropriate method
- Store the stock plasmid in an appropriate solution
  - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)
- Store the plasmid at –80ºC in single-use aliquots
- Assess plasmid quality with an agarose gel; a single band indicates high integrity plasmid, whereas a smear indicates degraded plasmid or excess enzymatic activity
- Assess the plasmid purity using a spectrophotometer for the following:
  - A260/A280 1.7–1.9 (lower ratios are indicative of contaminants from salts, carbohydrates, peptides, proteins, phenols, and guanidine thiocyanate)
  - Higher ratios may be indicative of RNA contamination

Assay Design Considerations

When using custom designed assays, several important considerations should be noted:

- Biological significance (correct isoform/splice variant chosen)
- Sequence quality and secondary structure — evaluate using web-based tools to understand the complexity of the structure, as it can impact the reaction performance
- Sequence length — use the entire gene sequence, or a specific region of interest, to optimally design an assay
- Sequence masking — use web-based masking tools to mask low complexity and repetitive regions to avoid assay design in these regions
- Uniqueness of the sequence — use BLAST or BLAT to ensure no homology exists and help avoid mispriming events
- Uniqueness of the assay — use *in silico* PCR, or Primer-BLAST, to "blast" the primers against the genome of interest to validate primer design specificity
- Default settings in the software — ensure they are set correctly (for example, salt conditions, oligo and amplicon sizes). The SsoAdvanced™ universal probes supermix and the qPCR cycling protocols have been optimized for assays with a primer melting temperature (T_m) of 60ºC designed using the open source Primer3, Primer3Plus or Primer-BLAST, default settings. For assays designed using other tools, the primer T_m should be recalculated using Primer3. **Suggested settings:** 50 mM Na^+, 3 mM Mg^{++}, 1.2 mM dNTPs, 250 nM annealing oligo, SantaLucia/SantaLucia

Some Key Design Considerations

- For optimal PCR efficiency, design the amplicon size between 70 and 150 bp (<70 bp may be needed for degraded/FFPE templates)
- Maintain primer lengths between 18 and 25 bp for good specificity and binding abilities
- Annealing temperatures between 58 and 62ºC are optimal (greater range can be obtained using Bio-Rad's Sso7d-based supermixes); temperatures >60ºC may result in less binding efficiency and <58ºC may result in less specificity
- The optimal amplicon GC content should be within 40–60% (greater range can be obtained using Bio-Rad's Sso7d-based supermixes)
- Avoiding primer secondary structures reduces potential primer-dimer issues
- Avoid mispriming by ensuring there are no more than 2 Gs or Cs in the last 5 bases on the 3' end of the primer
- Design your assay such that at least one primer or the probe spans an exon:exon junction site to avoid gDNA amplification
- Alternatively, design the assay such that the primers are in separate exons and the intron size is >1 kb
- Probe annealing temperature should be 8–10ºC higher than the primers to ensure binding to the template prior to extension
- Avoid placing Gs on the 5' end of the probe to avoid quenching of the fluorophore even after probe cleavage
- Probe lengths typically range from 18–30 bp, and vary depending on the type of probe chemistry used and the target sequence

Multiplex Assays Design Considerations

To ensure data generated in a multiplex reaction are equivalent to data generated in a singleplex reaction, it is imperative to evaluate the assay performance in both singleplex and multiplex reactions. It is also important to understand the expression level of your target sequences, as this will impact the multiplex optimization method.

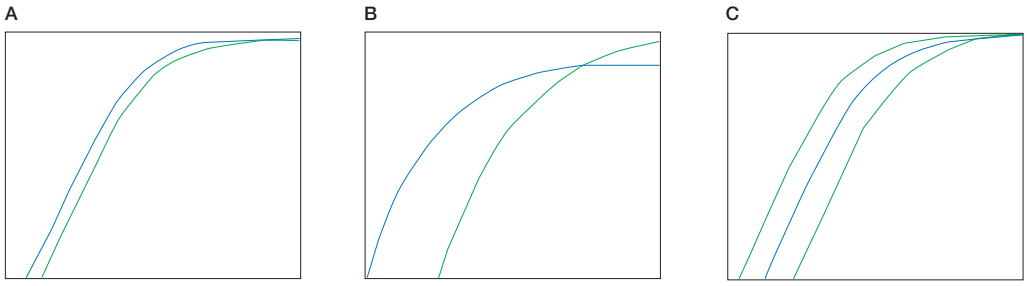


Fig. 1. Graphs show the three modes for expression in a duplex reaction. A, both genes express relatively equally; **B**, one gene always expresses more than the other; **C**, one gene varies in expression levels depending on the sample.

1. Determine the expression levels of the genes prior to optimizing a multiplex approach. This can be accomplished through the use of standard curves derived from template serial dilutions (for example 100 ng to 1 pg).
2. Assign the reporter dyes based on the expression levels; brighter fluorophores should be reserved for lower expressing targets.
3. Consider these factors when designing primers and probes:
   - All assays in a multiplex reaction should have the same or nearly the same annealing temperature
   - Analyze all oligos for primer-dimer stability with all other oligos in the reaction
   - Account for nonspecific primer-probe annealing and cross reaction between assays
   - Amplicons of 50–150 bases are preferred. Shorter amplicons often have better PCR efficiencies
4. Optimize each primer set.
   - Not all primer sets/concentrations perform the same. Empirical testing using a standard curve is imperative
   - Prepare two standard curves for each assay
     - One singleplex for each assay (if not completed already)
     - One duplex (or multiplex if applicable)
   - PCR efficiencies must be similar to minimize amplification bias
   - When combined in a reaction, be sure each assay is used within the appropriate template/target dynamic range. This range is often reduced when used in a multiplex application

5. Reduce the primer concentration of the higher expressing target.
   - Reducing the concentration, often times to 100–150 nM final, enables the lower expressing target to amplify sufficiently. Use a primer matrix to determine the optimal concentration, at which the chosen concentration would yield no shift in Cq values while exhibiting the lowest flwescence signal
6. Reduce the primer concentration of both or all targets when the expression levels are unknown or vary from sample to sample.

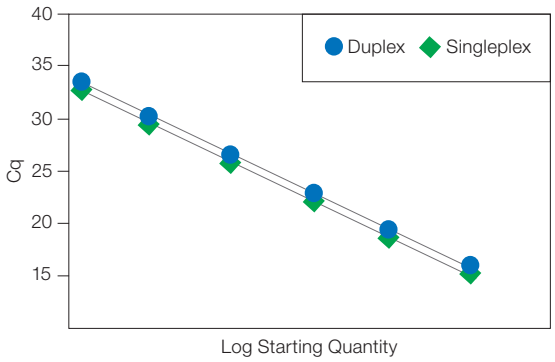


Fig. 2. Overlay of singleplex and duplex standard curves demonstrating no significant performance differences, thus a well-optimized duplex exists.

Notes: Preferential amplification of targets:

- Target abundance should not vary too much between assays; limiting variance guards against domination of the reaction by a more abundant target
- Verify that the Cq values have not changed between the singleplex and multiplex standard curves
  - If unavoidable, it may be permissible if all assays in the reaction shift equally

Tips to Get Started

- Always evaluate the performance of the supermix following the recommended reaction and cycling conditions prior to modification
- Be sure to set the activation time to 30 sec for cDNA and 2–3 min for genomic DNA
- The 2x supermix has been optimized for 20 µl reactions in 96-well plates and 10 µl reactions in 384-well plates

Procedure

Reaction Mix Preparation and Thermal Cycling Protocol

1. Thaw SsoAdvanced™ universal probes supermix and other frozen reaction components to room temperature. Mix thoroughly, centrifuge briefly to collect solutions at the bottom of tubes, and then store on ice protected from light.
2. Prepare (on ice or at room temperature) enough reaction setup for all qPCR reactions by adding all required components except the template according to the following recommendations (Table 1).

Table 1. Reaction setup.*

Component	Volume per 20 µl Reaction	Volume per 10 µl Reaction	Final Concentration
SsoAdvanced universal probes supermix (2x)	10 µl	5 µl	1x
Forward and reverse primers	Variable	Variable	250–900 nM each
Fluorogenic probe	Variable	Variable	150–250 nM each
Template (**add at step 4**)	Variable	Variable	cDNA: 100 ng–100 fg Genomic DNA: 500 ng–5
pg Nuclease-free H_2O	Variable	Variable	—
Total reaction mix volume	20 µl	10 µl	—

* Scale all components proportionally according to sample number and reaction volumes.

3. Mix the assay master mix thoroughly to ensure homogeneity and dispense equal aliquots into each PCR tube or into the wells of a PCR plate. Good pipetting practice must be employed to ensure assay precision and accuracy.
4. Add samples (and nuclease-free H_2O if needed) to the PCR tubes or wells containing the reaction setup (Table 1), seal tubes or wells with flat caps or optically transparent film, and vortex 30 sec or more to ensure thorough mixing of the reaction components. Spin the tubes or plate to remove any air bubbles and collect the reaction mixture in the vessel bottom.
5. Program thermal cycling protocol on the real-time PCR instrument according to Table 2.
6. Load the PCR tubes or plate onto the real-time PCR instrument and start the PCR run.
7. Perform data analysis according to the instrument-specific instructions.

Table 2. Thermal cycling protocol.

Real-Time PCR System	Setting/Scan Mode	Polymerase Activation and DNA Denaturation	Amplification		
			Denaturation at 95/98°C	Annealing/ Extension + Plate Read at 60°C**	Cycles
Bio-Rad® CFX96™, CFX384™, CFX96 Touch™, CFX384 Touch™, CFX Connect™	All channels	30 sec at 95°C for cDNA or 2–3 min at 95°C for genomic DNA*	5–15 sec	10–30 sec	35–40
Bio-Rad® iQ™5, MiniOpticon™, Chromo4™, MyiQ™	Standard			15–30 sec	
ABI 7500, StepOne, StepOnePlus, 7900HT and ViiA7	Fast Standard			10–30 sec 60 sec	
Roche LightCycler 480	Fast Standard			10–30 sec 60 sec	
Qiagen Rotor-Gene and Stratagene Mx series	Fast			10–30 sec	

* 2–3 min denaturation at 95°C is highly recommended for genomic DNA template to ensure complete denaturation.

** Shorter annealing/extension times (1–10 sec) can be used for amplicons <100 bp. Longer annealing/extension times (30–60 sec or more) can be used for amplicons >250 bp, GC- or AT-rich targets, low expressing targets, crude samples, or for higher input amounts (for example, 100 ng of cDNA or 500 ng of genomic DNA).

Real-Time PCR Validation for Gene Expression Experiments

The following validation experiments are critical for obtaining valid and publishable real-time PCR data following the MIQE guidelines. These simple-to-follow experiments should be completed prior to starting a new real-time PCR project.

Determining the Optimal Reference Gene

To properly perform a gene expression experiment, it is imperative that an optimal reference gene(s) is used. The reference gene(s) must maintain a consistent expression level across all samples in the project regardless of treatment, source, or extraction method. The variation in reference gene expression is somewhat dependent on the level of fold change discrimination desired. For example, if a twofold change in expression is important, then the reference gene should have little to no variation in expression. However, if a 20-fold change in expression is important, then the reference gene expression can have some variability. To validate a reference gene(s), follow the steps on the next page.

1. Begin searching for a candidate list of reference genes by searching publications, speaking with researchers using similar model systems, and mining microarray data, if available. Minimally, five reference genes should be selected for evaluation. For your convenience, Bio-Rad offers pre-plated reference gene panels using our highly validated and optimized PrimePCR assays.

2. From your experiment, randomly select a few samples from each group (for example, treatments, time courses, sources) ensuring that you evaluate all variable sample groups.
3. Isolate the RNA and DNase-treat using the same protocol for all samples. Quantify and normalize the RNA to the same concentration.
4. Perform a reverse transcription reaction for each sample using the same kit, volume, and concentration. Dilute the cDNA, as needed, treating each sample the same to ensure there are no differences from sample to sample in terms of volume and concentration from the initial RNA input.
5. Perform a real-time PCR experiment using the samples and the candidate reference genes using technical triplicates for each sample.
6. Evaluate the data for each reference gene by calculating a standard deviation for all samples. For example, if you evaluated eight samples and seven reference genes, simply calculate the standard deviation of those eight samples' Cq values for each reference gene. Thus, you will end up with seven standard deviation values. Compare the values to determine which reference gene(s) have the lowest value. Although there is no precise threshold for determining a good reference gene, a good rule of thumb is to ignore any reference gene with a standard deviation higher than 0.5. If you are using a Bio-Rad CFX real-time PCR system, you can utilize the software to automatically calculate an M-value to assist in determining the optimal reference gene.

   In this data set (Figure 3), TBP and PPIA are both below 0.5 and may be suitable reference genes for the given project. Keep in mind there is no one good reference gene for all projects, so the reference gene must be validated for every project.

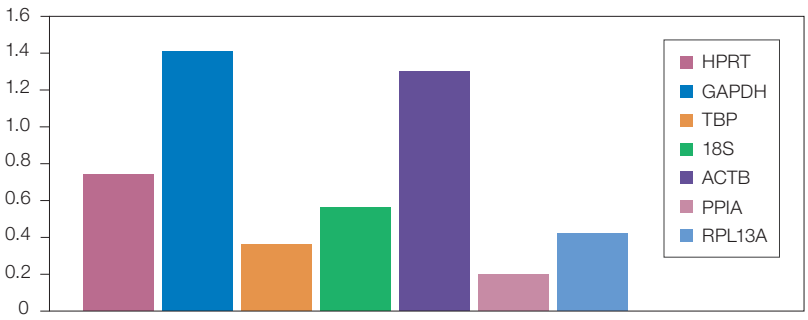


Fig. 3. Seven reference genes evaluated using random samples from untreated and treated sample groups. TBP and PPIA exhibited the lowest standard deviations with ~0.4 and 0.2, respectively. Note that GAPDH and ACTB exhibited the highest standard deviations, thus would be unacceptable reference genes. If you are unable to find a single stable reference gene, consider using multiple reference genes. This method involves calculating a geometric mean of the reference gene quantities (not Cq values) prior to performing the normalization.

Determining the Dynamic Range of the Reverse Transcription Reaction

An optimal reverse transcription reaction is expected to generate a true representation of the RNA converted into cDNA. However, it is imperative to determine the dynamic range of the reaction to ensure that the initial RNA loaded does not fall outside the dynamic range. If it does, then the downstream real-time PCR data may be invalid. To validate the dynamic range, perform the following :

1. Preparation of a serial dilution using a single RNA source (or a pooled RNA sample) is required to prepare the cDNA synthesis reactions for the experiment. Ensure an adequate amount of RNA is available; adjust concentrations and volumes accordingly.
2. Start with 1 µg of total RNA and perform a tenfold serial dilution covering at least 5 or 6 logs of dynamic range.
3. Perform RT using 20 µl reactions. Transfer the RNA, as shown in Figure 4, to the respective reaction tubes. For example, transfer 1 µg of RNA to Reaction 1 tube. Repeat transferring RNA to the remaining reaction tubes.

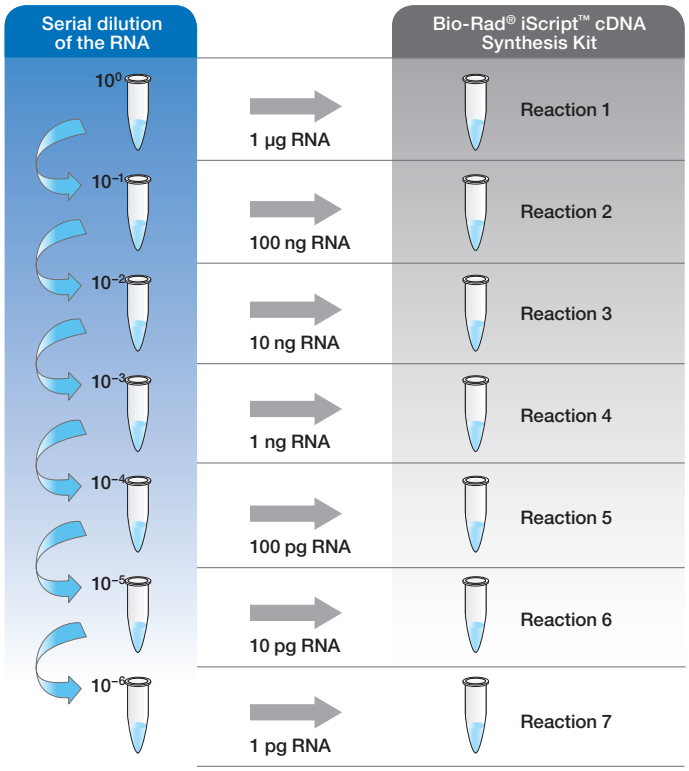


Fig. 4. Tenfold serial dilution of RNA starting at 1 µg down to 1 pg, thus covering six logs of dynamic range. Each RNA dilution was transferred to the respective cDNA reaction tube for cDNA synthesis.

4. Dilute the cDNA as needed to perform real-time PCR reactions using a minimum of two genes — reference and low expressing. However, it is recommended to evaluate four genes — reference, low, medium, and high expressing.
5. Prepare the real-time PCR plate (Figure 5) and cycle according to the recommended protocol.

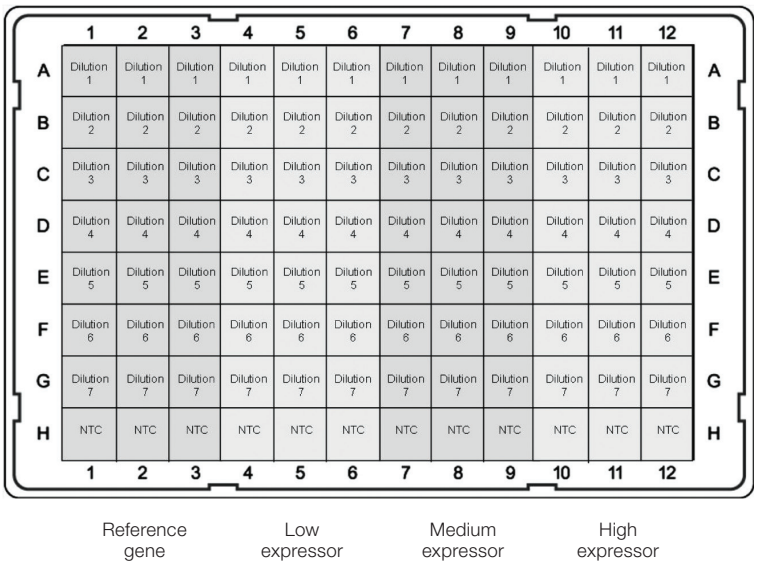


Fig. 5. A recommended plate layout.

6. Evaluate the data. Follow the guidelines in this manual (page 14–15) for setting the baseline and threshold prior to analyzing the data. Figure 6 illustrates the most common results from the experiment and how to interpret the data.

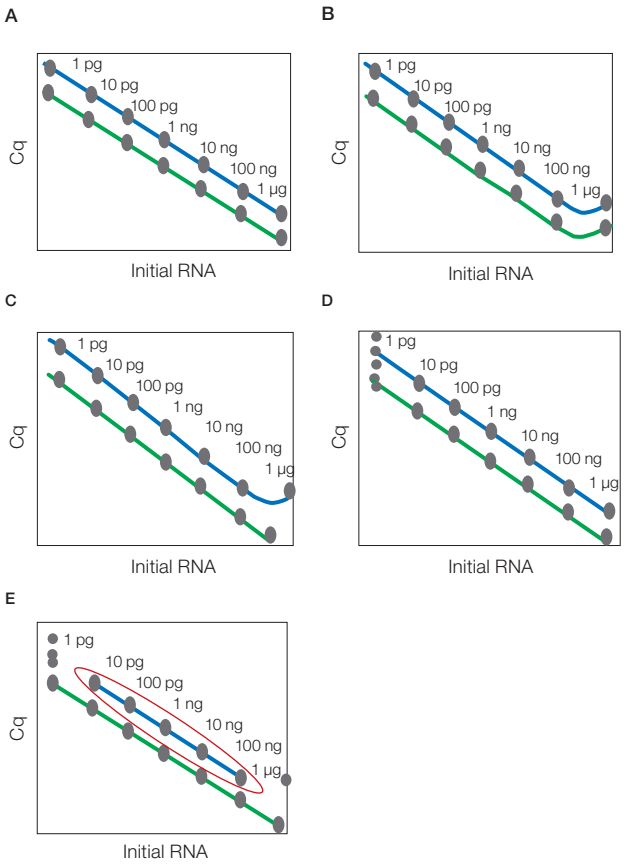


Fig. 6. The blue standard curve represents the target gene and the green standard curve represents the reference gene. A, both assays demonstrate equivalent performance in linearity and dynamic range covering 1 µg to 1 pg. Thus, any RNA input going forward within this range will be acceptable; **B**, both assays are either saturated at the 1 µg data point or the reverse transcription reaction is inhibited due to carryover inhibitors from the RNA sample. Consider using less RNA (≤100 ng) or re-purifying the RNA; **C**, the reference assay has a broader dynamic range than the target assay, therefore, the dynamic range is limited. Consider reevaluating the target assay design, using less RNA (≤100 ng), or re-purifying the RNA; **D**, the target assay exhibits a high standard deviation at the lowest concentration (1 pg) and should not be considered part of the dynamic range. This is due to a lack of sensitivity or reproducibility, and may be alleviated by using a carrier in the RNA sample such as glycogen or non-target gDNA carrier; **E**, after considering all the data, the concentration points that define the dynamic range from rejecting the variant 1 pg data and the saturated/inhibited 1 µg data point results in an effective dynamic range (RNA loading) is 1–100 ng.

Determining the PCR Efficiency

Determining the PCR efficiencies of your reference gene and target gene(s) is critical before starting any real-time PCR experiment. Knowing the PCR efficiency determines the appropriate relative gene expression math model. Not knowing may affect and invalidate the results. To determine the PCR efficiency among other key characteristics, prepare standard curves to evaluate the following:

- **PCR efficiency**
- Dynamic range
- Correlation coefficient
- Sensitivity
- Specificity

1. A serial dilution of the cDNA, gDNA, or plasmid template is required to prepare the standard curve. Ensure an adequate supply of template and an adequate volume are available to evaluate all the assays used in the experiment.

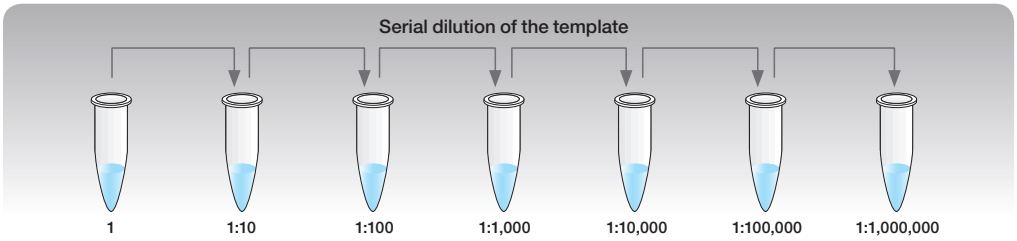


Fig. 7. Tenfold serial dilution covering 6 logs of dynamic range is prepared using a starting template of your choice based on target expression levels.

2. Prepare the real-time PCR reactions using a fresh bottle of supermix, nuclease-free water, and primer sets. Figure 8 is an example of a plate layout.

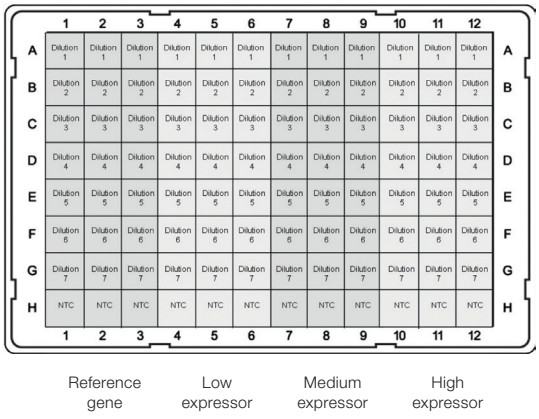


Fig. 8. Example of a plate layout with four seven-point standard curves with NTCs in technical triplicates — one for each gene of interest and the reference gene.

3. Cycle according to the recommended protocol.
4. Analyze the data. Follow the guidelines in this manual for setting the baseline and threshold prior to analyzing the data.
5. To determine which math model should be applied, simply subtract the slope value of the reference gene from each target gene. If the Δslope is ≤0.1, then the PCR efficiencies are within accepted limits and the $\Delta\Delta C_T$ math model can be used. If the Δslope is ≥0.1, then the efficiency correction math model (Pfaffl method) must be applied.

Tips for Success

- Pipet a minimum of 5 µl for each sample. This ensures greater precision and a smaller standard deviation for technical replicates. If the samples are too concentrated, simply dilute accordingly. Use a calibrated pipet of the appropriate volume range and never plunge the tip more than several millimeters below the surface of the sample. Pipet slowly and use the pipet tip demarcations to visualize accuracy
- Prepare individual master mixes for each sample by combining the real-time PCR supermix, nuclease-free water, and primers along with the template and mix thoroughly. Then, pipet 20 µl into the respective wells on the plate
- A tenfold dilution series is recommended to cover the most logs of dynamic range; however, depending on the expression level of the gene(s) evaluated and the total template amount available, this can be reduced to a fivefold dilution series

Efficiency

Calculate efficiency using the software or the following equation:

$E = 10^{[-1/m]} - 1$. A PCR efficiency from 90–110% (slope values from –3.6 to –3.1) is preferred.

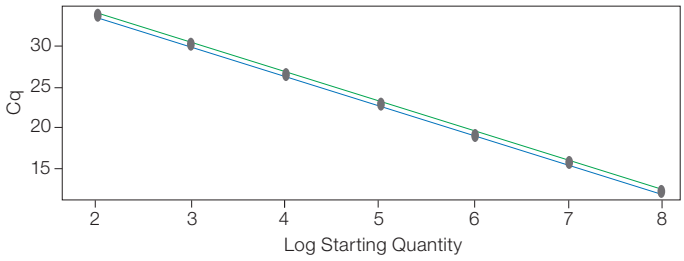


Fig. 9. Reference gene has a PCR efficiency of 97.59% (–3.381) and six logs of dynamic range. The target gene has a PCR efficiency of 99.17% (–3.342) with six logs of dynamic range. Subtracting the slope values, 3.381 – 3.342 = 0.039, which is <0.1.

Linearity

Calculate the R^2 statistic for each standard curve using the qPCR analysis software; the R^2 should be ≥–0.980. However, if the R^2 is <0.980, remove outliers. If there are too many outliers, then reevaluate the experiment to determine the cause of the lower R^2 value.

Dynamic Range

Determine the general trend of the slope where linearity (R^2) and efficiency are within acceptable ranges, as specified above.

Sensitivity

Determine the lowest concentration of the serial dilution where replicate reproducibility is high and the R^2 of the standard curve is ≥0.980.

Specificity

For probe-based assays, achieving optimal specificity requires observing a single band (PCR product) following a gel analysis.

Troubleshooting Guide

Poor Nucleic Acid Yields

Review Tables 3 and 4 to determine if you are within an acceptable range of nucleic acid yield. If your yields of RNA are considerably less than is typical for your sample type, reevaluate your isolation method. For reference, typical yields from some mammalian tissues are listed in tables 3 and 4.

Table 3. RNA yields.

Total RNA per Cell	Total DNA per Cell
5–30 pg	Varies by genome

Table 4. RNA yields per mg of tissue.

Sample Type	Yield
Liver	4 µg
Spleen	4 µg
Heart	3 µg
Kidney	2 µg
Lung	2 µg
Brain	1.5 µg
Bone	50 ng
Adipose	<10 ng

PCR Inhibitors/Oversaturation

If you suspect that your sample(s) contains PCR inhibitors, consider the following corrective actions:

1. Evaluate your sample type to determine if any of the common inhibitors listed in the following list may be present in your sample as carryover. If you suspect contamination, re-purify the samples using a commercially available post-isolation cleanup kit.
2. Evaluate the A260/280 and A260/230 ratios. Refer to the RNA/DNA isolation section (page 1–2) in this manual for further information.

Common PCR Inhibitors*

From the Sample	From the Isolation Method
Melanin	EtOH >1% v/v
Polysaccharides	Proteinase K
Polyphenolics	DMSO >5%
Hemoglobin	EDTA >50 mM
Chlorophyll	SDS >0.01% w/v
Heparin	Sodium Acetate >5 mM
Humic acid	Mercaptoethanol
Hematin	Guanidinium
	Phenol >0.2% v/v
	DTT >1 mM

* Not an inclusive list.

3. If the most concentrated sample in the dilution series is showing compression, as seen in Figure 10, where the tenfold dilution series ΔCq value is < 3.3 compared to the more diluted points, then PCR inhibitors are most likely present in the sample. However, compression may also be due to an overloaded amount of template, error in the dilution series, or pipetting error.
   a. Re-purify the sample(s) using a different isolation method, or post-isolation column cleanup
   b. Remove the highest dilution point
   c. Increase the annealing/extension time

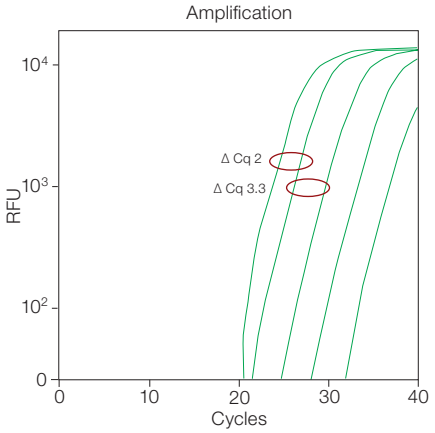


Fig. 10. Presence of PCR inhibition at the highest dilution point, as indicated by delayed amplification.

Low Template Input, Low Expression, High Cq Values

If your Cq values are higher than expected or you are concerned about Cq values >30, consider the following corrective actions:

1. Confirm the expected expression level, if known, to ensure that the target of interest is present in your given sample. Additionally, consider higher input concentrations of sample for low expressing targets. Remember that for every twofold increase in starting sample concentration, the Cq value shifts one cycle earlier (assuming 100% PCR efficiency).
2. Confirm the template input amount using a fluorescence-based quantification method to ensure the cDNA. input range is 100 ng to 100 fg or the genomic DNA input range is 500 ng to 5 pg. (cDNA will require purification prior to quantification analysis.)
3. Increase the volume of template pipetted into the PCR reaction. For the highest accuracy and precision, pipet a minimum volume of 5 µl for each sample.
4. Consider adding a carrier to your sample stock to increase homogeneity — examples include tRNA, glycogen, and unrelated gDNA.
5. Consider using nonstick polypropylene tubes for sample stock storage to prevent nucleic acid from binding to the tube walls.
6. Confirm that the reverse transcription reaction was successful. A simple-to-follow protocol is outlined in Reagent Comparison Guide for Real-Time PCR

Amplification Plots

If you notice that any data point(s) in your amplification plots exhibit a sigmoidal shape in the log view (Figure 11, left), this is typically due to an incorrect baseline setting. Consider the following corrective actions:

1. Deselect automatic baseline setting and assign manual baseline. Adjust the baseline begin and end cycles so that the amplification plot matches the others on the plot. Sometimes this takes a few tries, but a general rule of thumb is to set the end cycle about two cycles before the start of true amplification, as seen in Figure 12.

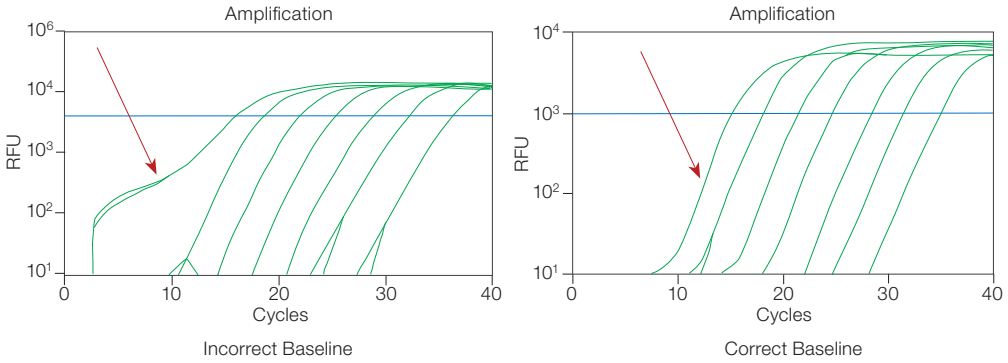


Fig. 11. Incorrect baseline is exhibited in the left graph indicated by the arrow pointing to the first dilution point where the amplification plot is more sigmoidal in shape. As a result, an artificially lower Cq value is obtained. Corrected baseline is shown in the graph on the right.

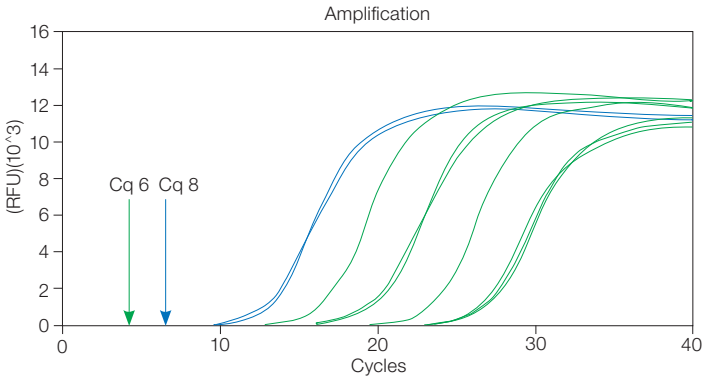


Fig. 12. Baseline setting is best completed in the linear view. In this example, the amplification starts around cycle 8; therefore, setting the end baseline two cycles prior at cycle 6 is best.

2. Either remove this data point or dilute your sample so that it does not show amplification earlier than cycle 15. This ensures that the software's algorithm has enough background to subtract from the signal. Early amplification may cause the algorithm to fail due lack of background data.

If you notice high standard deviations for technical replicates or inconsistent gene expression data, this could be due to the threshold being positioned either too high or too low. Consider the following corrective action:

When setting the threshold, you should choose a position that is in the middle of the geometric (exponential) phase of PCR. Setting the threshold too high or too low places the threshold in a less than ideal region of amplification where greater noise is present and PCR is not 100% efficient.

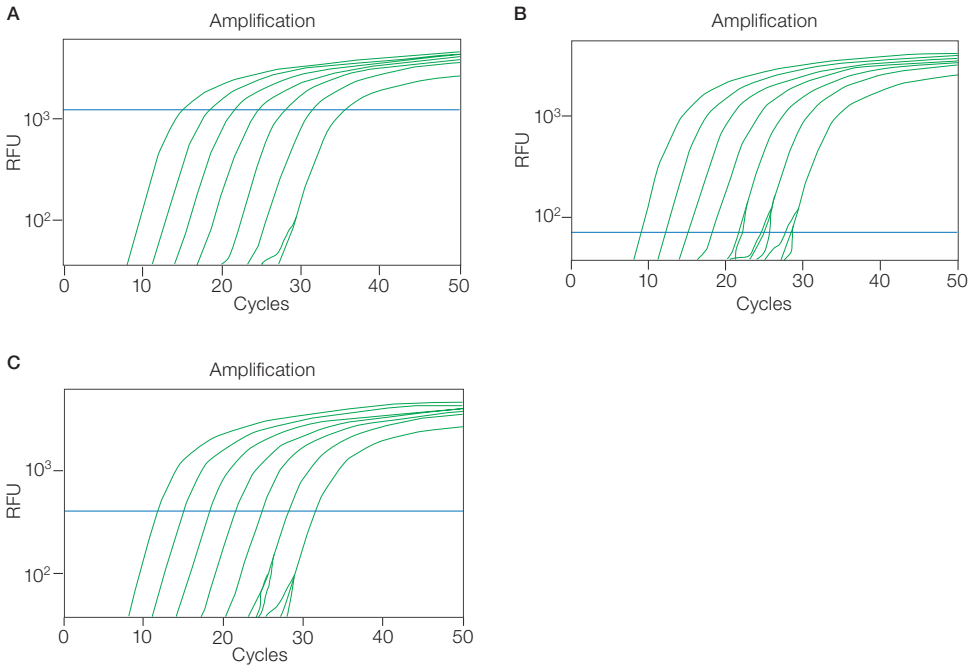


Fig. 13. Illustrations of baseline settings. A, when the threshold is set too high, the data collected are often from the linear phase of PCR, where the reaction is not the most efficient; **B**, the threshold is set too low. When set too low, the data collected are often within the background noise of the reaction; **C**, a correct threshold setting where the data collected are within the geometric (exponential) phase of PCR.

PCR Performance Not 100% Efficient

If you have already ruled out your samples as a source for poor efficiency, then the assay may be the cause of the problem. Please review the section on assay design in this manual for further information (page 3).

Also, consider the following corrective action:

Perform a temperature gradient experiment to determine the optimal annealing temperature. Set up the gradient as follows:

a. Use several representative samples in your project.

b. Set the temperature range 10ºC above and 6ºC below the calculated annealing temperature.

c. Choose the final annealing temperature based on overall performance related to specificity.

1. Evaluate the assay design by following the bioinformatics workflow outlined in the beginning of this manual (page 3). This will help ensure that the primers are highly specific to your target of interest and no other target region(s).
2. Perform a temperature gradient to determine the optimal annealing temperature of the primers. Load your plate with the same reaction setup and sample for each primer set in a column format so that you can evaluate the annealing temperatures. Set the gradient 10ºC above and 6ºC below the calculated annealing temperature to ensure a proper temperature range is covered. Choose the best temperature based on the overall PCR amplification, keeping in mind that lower temperatures may reduce specificity and higher temperatures may reduce primer binding efficiency.

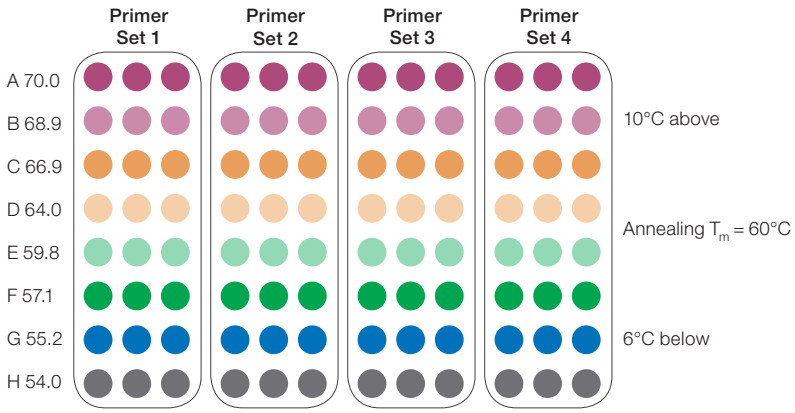


Fig. 14. Temperature gradient layout.

If you suspect the standard curve and dilution points are not within the MIQE guidelines of 90–110% PCR efficiency with an R^2 of 0.99 or greater, consider the following corrective actions:

1. Ensure that the standard curve covers at least 5–6 logs of dynamic range. When the standard curve is too small, the variability of the true efficiency greatly increases.
2. If the R^2 is <0.98, review the standard curve data points for outliers. Remove any outliers where the ΔCq is >0.5 for the group. For example, if your 100 pg dilution point has Cq values of 29.2, 29.6, and 30.5, you should remove the Cq value of 30.5. If there are too many outliers, it may be a sign of other technical issues.

Control Samples/Wells Are Not Performing as Expected

If your non-template control (NTC) wells indicate amplification, you need to determine the source. Although the most likely cause is nucleic acid contamination, other possible causes include:

- Pipetting template into the NTC well
- Sample from adjacent wells being aerosolized while pipetting or removing the plate seal after samples have been loaded
- Contaminated plate, water, primers, or supermix
- Use of nonfiltered pipet tips
- Degraded probe

1. Evaluate your current workflow and adjust as needed. If you suspect your reagents are contaminated, the best method to determine the source is to replace them one at a time starting with the water, which is a common source of contamination. Next, make a fresh dilution of primers from the stock solution. And finally, use a new aliquot of the supermix. Discard any identified contaminated reagent from the lab.
2. If the problem persists, evaluate the background noise for the entire real-time PCR run across all wells. If the signal is unusually high compared to prior runs, your probe may be degrading. When this occurs, the high temperatures cause the probe to cleave, thus releasing the reporter dye into solution and allowing fluorescence. Probes should be aliquoted upon receipt into amber tubes and should not be exposed to freeze/thaw cycles > five times, as this causes premature degradation.

If your no-RT control wells indicate amplification, you need to determine the amount of gDNA contamination present in your cDNA sample(s) to understand the impact on your data.

1. Using Table 5, determine the percent of gDNA contamination present. For example, if the ΔCq (no-RT control Cq – cDNA Cq) for a given sample is seven or greater, then less than 1% of the DNA present in the sample is gDNA, which would be considered insignificant.

Table 5. Determining percent of gDNA contamination.

ΔCq	Percent Contribution, %
1	50.00
2	25.00
3	12.50
4	6.25
5	3.125
6	1.5625
7	0.78125

2. Evaluate the assay design and note the location of the primers. To avoid gDNA amplification, at least one primer must span an exon:exon junction site. Alternatively, the primers can be designed in two different exons that are separated by an intronic region >1 kb.

If you are using an internal positive control (IPC) and the standard deviation of the Cq values across all samples is >0.167, then consider the following:

When the IPC for a given sample(s) is higher than the group, this is most likely due to the presence of a PCR inhibitor. Review the sections on sample preparation for more information.

Multiplexing

Ideally, data between singleplex and multiplex should remain the same in terms of Cq values and PCR efficiency. In addition, if your data exhibits relative Cq shifts for all data points between singleplex and multiplex, then the final data output remains the same. However, if you observe variable Cq shifts for respective data points between singleplex and multiplex, consider the following:

The higher expressing assay may be using up the reaction components such as dNTPs and enzyme, and thus causing a shift in the lower expressing assay to later Cq values than observed in singleplex.

1. Primers and probes from different assays may be interacting. Make sure there are no stable dimers formed between the oligos from different assays. This can be completed using various open source tools online.

2. If the lower expressing assay has a longer amplicon, >150 bp, then consider redesigning the assay to be shorter or equivalent in length to the higher expressing gene. Shorter amplicons typically can have greater PCR efficiencies.
3. Choose assays with more similar expression levels, if possible, to avoid reagent competition. If this strategy is not possible, optimize the assays using a primer-limiting strategy to limit the available primer for the higher expressing gene. This in turn forces an earlier plateau phase of PCR.
   a. Construct a primer matrix (Table 6.) for the higher expressing assay ranging from 50 nM to 150 nM while keeping the lower expressing assay constant.
   b. Select the concentration that generates the lowest fluorescence signal without any effect on the Cq compared to singleplex data.
   c. Repeat the multiplex experiment to compare the newly optimized primer set.

Table 6. Primer matrix.

	Forward Primer, nM		
Reverse Primer, nM	**50**	**100**	**150**
50	50/50	100/50	150/50
100	50/100	100/100	150/100
150	50/150	100/150	150/150

Ordering Information

Catalog #	Description
SsoAdvanced Universal Probes Supermix	
172-5280	2 ml (2 x 1 ml vials), 200 x 20 µl reactions
172-5281	5 ml (5 x 1 ml vials), 500 x 20 µl reactions
172-5282	10 ml (10 x 1 ml vials), 1,000 x 20 µl reactions
172-5284	25 ml (5 x 5 ml vials), 2,500 x 20 µl reactions
172-5285	50 ml (10 x 5 ml vials), 5,000 x 20 µl reactions
Two-Step Reverse Transcription Reagents	
170-8842	**iScript Advanced cDNA Synthesis Kit for RT-qPCR**, 50 x 20 µl reactions
170-8843	**iScript Advanced cDNA Synthesis Kit for RT-qPCR**, 250 x 20 µl reactions
170-8890	**iScript cDNA Synthesis Kit**, 25 x 20 µl reactions
170-8891	**iScript cDNA Synthesis Kit**, 100 x 20 µl reactions
170-8840	**iScript Reverse Transcription Supermix for RT-qPCR**, 25 x 20 µl reactions
170-8841	**iScript Reverse Transcription Supermix for RT-qPCR**, 100 x 20 µl reactions
170-8896	**iScript Select cDNA Synthesis Kit**, 25 x 20 µl reactions
170-8897	**iScript Select cDNA Synthesis Kit**, 100 x 20 µl reactions
PCR Plate Sealers	
MSA-5001	**Microseal 'A' Film**, package of 50 seals
MSB-1001	**Microseal 'B' Adhesive Seals**, optically clear, 100 seals
MSC-1001	**Microseal 'C' Optical Seals**, 100 seals

Description	Clear Wells	White Wells	Black Wells
Hard-Shell Low-Profile 96-Well Skirted PCR Plates			
White shell, 50	HSP-9601	HSP-9655	—
Red shell, 50	HSP-9611	—	—
Yellow shell, 50	HSP-9621	—	—
Blue shell, 50	HSP-9631	HSP-9635	—
Green shell, 50	HSP-9641	HSP-9645	—
Black shell, 50	HSP-9661	HSP-9665	HSP-9666
White shell, bar-coded, 50	HSP-9901	HSP-9955	—
Hard-Shell High-Profile 96-Well Semi-Skirted PCR Plates			
Clear shell, 25	HSS-9601	—	—
Green shell, 25	HSS-9641	—	—
Black shell, 25	—	HSS-9665	—
Clear shell, bar-coded, 25	HSS-9901	—	—
Hard-Shell 384-Well Standard PCR Plates			
Clear shell, 50	HSP-3801	HSP-3805	—
Red shell, 50	HSP-3811	—	—
Yellow shell, 50	HSP-3821	—	—
Blue shell, 50	HSP-3831	—	—
Green shell, 50	HSP-3841	—	—
Black shell, 50	—	HSP-3865	HSP-3866
Clear shell, bar-coded, 50	HSP-3901	HSP-3905	—
Hard-Shell 384-Well 480 PCR Plates with Bar Code on Row A Side			
Clear shell, 50	HSR-4801	HSR-4805	—
Clear shell, 100 (2 packs)	HSR-4801K	HSR-4805K	—

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