Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix

Instruction Manual

Catalog #600886 (single kit)
#600887 (10-pack kit)
Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

600886-12

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Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix

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Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix

MATERIALS PROVIDED

Catalog #600886 (single kit), #600887 (10-pack kit)

Materials Provided	Quantity [a,b]
2× Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix	2 × 2 ml
RT/RNase Block	400 µl
100 mM DTT	100 µl
Reference Dye[c], 1 mM	100 µl

[a] Sufficient reagents are provided for four hundred, 20-µl QRT-PCR reactions.

[b] Quantities listed are for a single kit. For 10-pack kits, each item is provided at 10 times the listed quantity.

[c] The reference dye is light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All Components: Store at –20°C upon receipt. After thawing, the 2× master may be stored at 4°C for up to three months or returned to –20°C for long term storage

Note *The reference dye and master mix are light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

NOTICES TO PURCHASER

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Notice to Purchaser: Limited License

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Revision C

INTRODUCTION

Quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The Brilliant III SYBR® Green Ultra-Fast QRT-PCR Master Mix was developed for the ABI StepOnePlus and Bio-Rad CFX96 real-time PCR instruments and other fast-cycling systems (such as the ABI 7900HT and 7500 Fast systems). It performs QRT-PCR in less time without compromising target detection sensitivity, specificity, or reproducibility. The master mix includes two key components that enable it to perform optimally under fast cycling conditions:

- A mutated form of *Taq* DNA polymerase that has been specifically engineered for faster replication
- An improved chemical hot start mechanism that promotes faster hot start release to improve amplification specificity while keeping the run time of the PCR protocol to a minimum

The kit includes the components necessary to carry out cDNA synthesis and PCR amplification in one tube and one buffer.* The single-step master mix format is ideal for most high-throughput QPCR applications where it is not necessary to archive cDNA.

Features of Kit Components

RT/RNase Block

The reverse transcriptase (RT) provided in the kit is a Moloney-based RT specifically formulated for the Stratagene Brilliant III Ultra-Fast kits. This RT performs optimally at a reaction temperature of 50°C when used in 1-step QRT-PCR with the Brilliant III master mix. It is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis and to ensure sensitive and reproducible performance in QRT-PCR experiments with a broad range of RNA template amounts and a variety of RNA targets that vary in size, abundance, and GC-content. The RNase block, provided in the same tube, serves as a safeguard against contaminating RNases.

Brilliant III Ultra-Fast SYBR Green QRT-PCR 2× Master Mix

The 2× master mix contains an optimized RT-PCR buffer, $MgCl_2$, nucleotides (GATC), stabilizers, SYBR Green I dye and mutant *Taq* DNA polymerase. The DNA polymerase features a rapid hot start capability that reduces nonspecific product formation.

DTT

A separate tube of 100 mM DTT is provided with the kit. Adding DTT to the reactions improves RT performance for more challenging targets.

* Primers and template are not included.

Reference dye

A passive reference dye (an optional reaction component) is provided in the kit as an optional reagent that may be added to compensate for non-PCR related variations in fluorescence. Providing the reference dye in a separate tube makes the Brilliant III QRT-PCR kit adaptable for many real-time QPCR platforms (see *Reference Dye* in *Preprotocol Considerations* for more information).

Fluorescence Monitoring in Real-Time

When fluorescence signal from a PCR reaction is monitored in real-time, the results can be displayed as an amplification plot (see Figure 1), which reflects the change in fluorescence during cycling. This information can be used to quantify initial copy number based on threshold cycle (Ct).[1] Ct is defined as the cycle at which fluorescence is determined to be statistically significant above. In the amplification plot in Figure 1, for example, the Ct of each reaction is the cycle number at which the plot crosses the threshold line. The threshold cycle has been shown to be inversely proportional to the log of the initial copy number.[1] The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background. Quantitative information based on threshold cycle is more accurate than information based on endpoint determinations as Ct is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents and small differences in reaction components or cycling conditions.

In Figure 1, the Brilliant III Ultra-Fast SYBR Green QRT-PCR master mix was used in reactions containing serially diluted RNA template and a no-template control reaction (NTC) to amplify the GAPDH target. In the amplification plot (top panel) the reactions containing template show a significant increase in fluorescence with Ct values ranging from 15 to 36. The Ct values obtained in the amplification plot are used to generate the standard curve in the bottom left panel. In the dissociation/melt curve (bottom, right), PCR samples were subjected to a stepwise increase in temperature from 60°C to 95°C with fluorescence measurements taken throughout this range. The first derivative of fluorescence is then plotted versus temperature. As the temperature increases, the amplification products in each tube melt according to their composition. In Figure 1, the melt curve shows fluorescence peaks centered at 83°C, which correspond to the desired product in the reactions. If primer-dimer or nonspecific products had been made during the amplification step, they would generally melt at a different temperature (defined as the Tm) than the desired products.

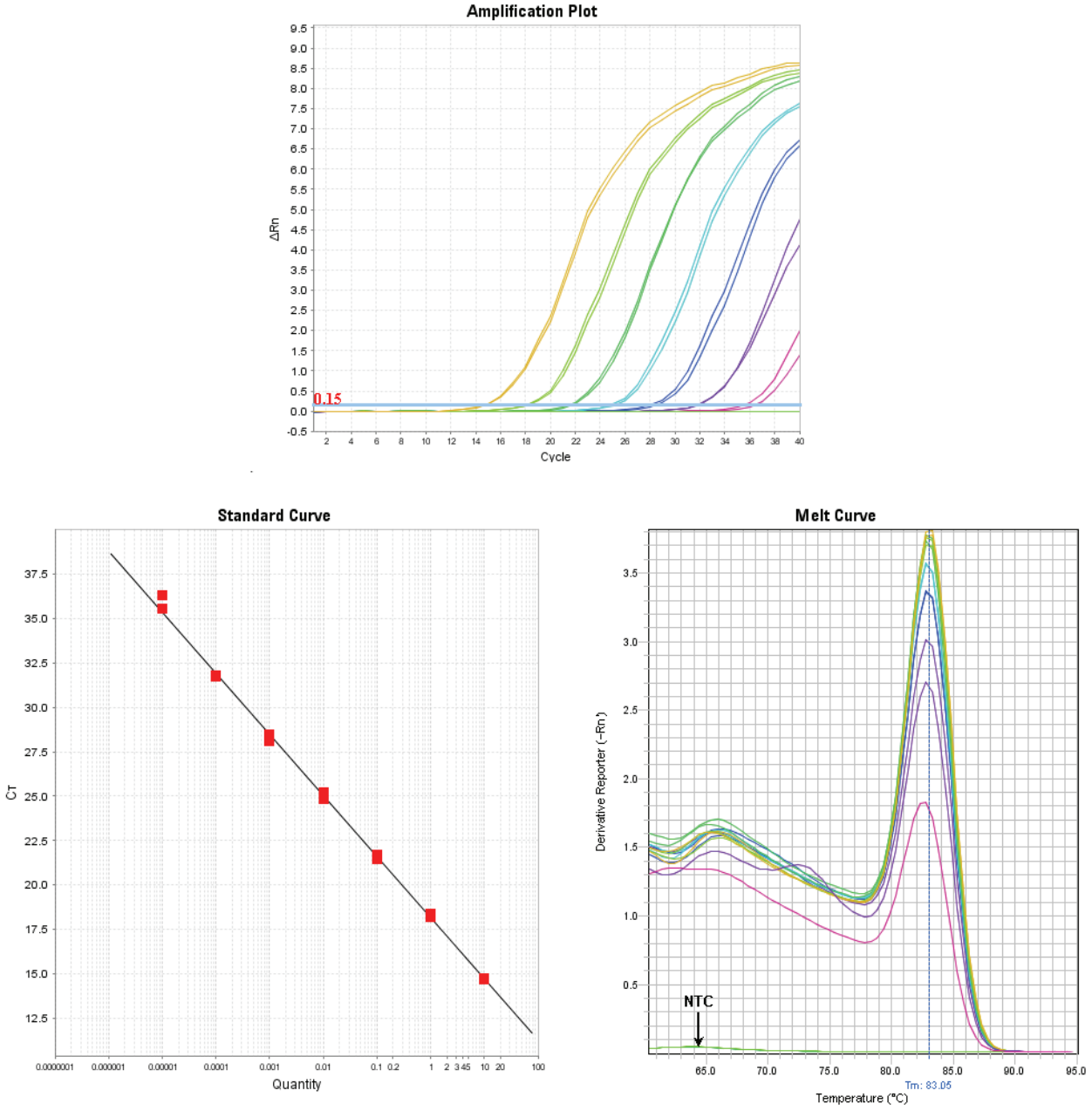

Figure 1 *Top panel*: StepOnePlus instrument amplification plot. A serial dilution of RNA template was added (ranging from 0.01 pg to 10 ng) to each reaction (set up in duplicate). The fluorescence value used to determine Ct (the threshold line) is shown as a solid line. *Bottom left panel*: Standard curve generated from amplification plot. An amplification efficiency of 95.36% and an R-squared value of 0.995 were obtained. *Bottom right panel*: Melt curve generated when amplified products were subjected to dissociation analysis. The fluorescence peaks corresponding to the amplicon are centered around 83.05°C.

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Total and poly(A)$^+$ RNA can be rapidly isolated and purified using Stratagene Absolutely RNA isolation kits. Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. RNA samples with $OD_{260/280}$ ratios of 1.8–2.0 are desired for QRT-PCR.

Preventing RNase Contamination

Take precautions to minimize the potential for contamination by ribonucleases (RNases). RNA isolation should be performed under RNase-free conditions. Wear gloves and use sterile tubes, pipet tips, and RNase-free water. Do not use DEPC-treated water, which can inhibit PCR. The RNase inhibitor that is included in the tube of RT/RNase Block provides additional protection against RNase contamination.

Preventing Genomic DNA Contamination

Contaminating DNA can be removed from the RNA preparation using an RNase-free DNase. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing genomic DNA.

Quantitative PCR Human Reference Total RNA

Stratagene Quantitative PCR (QPCR) Human Reference Total RNA (Catalog #750500) is a high-quality control for quantitative PCR gene-expression analysis. QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines (see the table below), with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The reference RNA is carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

QPCR Human Reference Total RNA Cell Line Derivations
Adenocarcinoma, mammary gland
Hepatoblastoma, liver
Adenocarcinoma, cervix
Embryonal carcinoma, testis
Glioblastoma, brain
Melanoma, skin
Liposarcoma
Histiocytic lymphoma; macrophage; histocyte
Lymphoblastic leukemia, T lymphoblast
Plasmacytoma; myeloma; B lymphocyte

The QPCR Human Reference Total RNA is ideally suited for optimizing SYBR Green QRT-PCR assays. Often only small amounts of experimental RNA template are available for setting up an expression profiling study. Using the extensive representation of specific mRNA species in the generic template, assays may be optimized for a variety of targets. This eliminates the use of precious experimental RNA samples for assay optimization.

RT-PCR Primer Concentration

It is critical to minimize the formation of nonspecific amplification products when performing single-step QRT-PCR reactions using SYBR Green detection. This issue becomes more prominent at low target concentrations.

Use the lowest concentration of primers possible without compromising the efficiency of PCR. The optimal concentration of the upstream and downstream PCR primers is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration, with minimal or no formation of primer-dimer. This concentration should be determined empirically, but primer concentrations in the range of 150–500 nM are generally satisfactory (with especially difficult targets, higher primer concentrations may improve detection).

Preventing Sample Contamination

Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips.

Magnesium Chloride

The optimal $MgCl_2$ concentration promotes maximal amplification of the specific target amplicon with minimal nonspecific products and primer-dimer formation. High levels of the Mg^{2+} ion tend to favor the formation of nonspecific dsDNA, including primer-dimers. Therefore, when a SYBR Green-based QPCR assay is being optimized, the $MgCl_2$ levels should be as low as possible, as long as the efficiency of amplification of the specific target is not compromised (typically between 1.5 and 2.5 mM $MgCl_2$). The Brilliant III SYBR Green QRT-PCR master mix contains $MgCl_2$ at a concentration of 2.5 mM (in the 1× solution), which is suitable for most targets. The concentration may be increased, if desired, by adding a small amount of concentrated $MgCl_2$ to the 1× experimental reaction at the time of set up.

Reference Dye

A passive reference dye is included in this kit and may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm, respectively. Although addition of the reference dye is not required when using the Bio-Rad CFX96 real-time PCR system, with other instruments (including the ABI StepOnePlus instrument) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If using a StepOnePlus or 7900HT Fast instrument, dilute the dye 1:50 for a final concentration of 300 nM in the reactions. For the Stratagene Mx instruments or the ABI 7500 Fast instrument, dilute the dye 1:500 for a final concentration of 30 nM. The Bio-Rad CFX96, the Roche LightCycler® 480 and the QIAGEN Rotor-Gene Q instruments do not require the use of the reference dye.

Reaction Preparation

Setting Up Reactions on Ice

While setting up the reactions, keep the reagent mixture and reaction tubes on ice until the reactions are loaded into the instrument.

Preparing a Single Mixture for Multiple Samples

If running multiple samples containing the same primers, we recommend preparing a single mixture of reaction components and then aliquoting the mixture into individual reaction tubes using a fresh pipet tip for each addition. Preparing a common mixture facilitates the accurate dispensing of reagents, minimizes the loss of reagents during pipetting, and helps to minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Solutions that contain enzymes (including reverse transcriptase and DNA polymerase) should be mixed gently by inversion or gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the buffer can lead to pipetting errors.

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the day for setting up additional assays.

Temperature and Duration of cDNA Synthesis Reaction

For cDNA synthesis, we recommend a 50°C incubation for use with the Brilliant III Ultra-Fast SYBR Green QRT-PCR master mix. A 10-minute incubation for the first-strand synthesis reaction is sufficient for most targets.

Recommended Control Reactions

No-Template Control (NTC)

We recommend performing no-template control reactions for each experimental sample to screen for contamination of reagents or false amplification.

No-RT Control

We recommend performing no-RT control reactions for each experimental sample by omitting the RT/RNase block from the reaction. The no-RT control is expected to generate no signal if there is no amplification of genomic DNA. No signal indicates that the RNA preparation is free of contaminating genomic DNA or that the primers are specific for the cDNA. See *Preventing Genomic DNA Contamination* in *RNA Isolation*.

Endogenous Control

Consider performing an endogenous control reaction to normalize variation in the amount of RNA template across samples. See Reference 2 for guidelines on the use of endogenous controls for QPCR.

Data Acquisition with a Spectrofluorometric Thermal Cycler

Acquisition of real-time data generated by fluorogenic dye should be performed as recommended by the instrument's manufacturer. Collect fluorescence data during the combined annealing/extension step.

PROTOCOL

Notes *Once the tube containing the 2× QRT-PCR master mix is thawed, store it on ice while setting up the reactions. Following initial thawing of the master mix, store the unused portion at 4°C for up to three months or return to –20°C for long term storage.*

SYBR Green I dye (present in the master mix) is light-sensitive; solutions containing the master mix should be protected from light whenever possible.

It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification. Similarly, a no-RT control should be included to verify that the fluorescence signal is due to the amplification of cDNA and not of contaminating genomic DNA.

Consider performing an endogenous control reaction to normalize variations in the amount of RNA template across samples. For information on the use and production of endogenous controls for QPCR, see Reference 2.

Preparing the Reactions

1. If using the reference dye, dilute the provided dye using nuclease-free PCR-grade H_2O. For the ABI StepOnePlus instrument or the ABI 7900HT Fast instrument, dilute the dye **1:50** (for a final concentration of 300 nM in the reactions). For the Stratagene Mx3000P or Mx3005P instrument or the ABI 7500 Fast instrument, dilute the dye **1:500** (for a final concentration of 30 nM in the reactions). **Keep all solutions containing the reference dye protected from light.**
2. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for replicate experimental reactions and replicate no-template-controls (plus at least one reaction volume excess), using multiples of each component listed below. *Keep the reagent mixture on ice.*

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 20 µl (including experimental RNA)
10 µl of 2× SYBR Green QRT-PCR master mix
x µl of upstream primer (150–500 nM final concentration)
x µl of downstream primer (150–500 nM final concentration)
0.2 µl of 100 mM DTT
0.3 µl of the **diluted** reference dye (optional)
1 µl of RT/RNase block

3. Mix the reagents well without creating bubbles, then distribute the mixture to individual PCR reaction tubes. *Keep the reactions on ice.*

4. Add *x* µl of experimental RNA to each reaction. The quantity of RNA depends on the RNA purity and the specific mRNA abundance. As a guideline, use 0.1 pg–100 ng of total RNA or 0.1 pg–1 ng of mRNA.

5. Gently mix the reactions without creating bubbles, then centrifuge the reactions briefly.

RT-PCR Cycling Program

6. Place the reactions in the instrument. Based on the instrument you are using, select the appropriate PCR program from the tables below. Set the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	12 seconds	60°C

ABI 7900HT Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	15 seconds	60°C

ABI StepOnePlus

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

QIAGEN Rotor-Gene Q

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	10 seconds	95°C
	10–20 seconds*	60°C

Roche LightCycler® 480

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

Stratagene Mx3000P and Mx3005P

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds*	95°C
	20 seconds	60°C

* The exact duration needs to be optimized for each target. Challenging templates (e.g. GC-rich RNA or RNA with extensive secondary structure) generally require longer denaturation and annealing/extension times than low-complexity templates.

Dissociation Program

7. For your specific instrument, follow the manufacturer's guidelines for generating dissociation curves.

TROUBLESHOOTING

Observation	Suggestion(s)
No (or little) increase in fluorescence with cycling in the amplification plots	Ensure that the correct amount of template was used and that the template sample is of good quality. If unsure, make new dilutions of template before repeating PCR. It may also be helpful to check for PCR inhibitors by adding this target into an assay that is known to work.
	The RNA template sample may contain inhibitors of PCR. Use the Stratagene Alien QRT-PCR Inhibitor Alert to test the sample for the presence of inhibitors.
	The target is highly GC-rich. Raise the denaturation temperature to 98°C or titrate DMSO into the reactions in 1% increments.
	Taq DNA polymerase was not activated. Ensure that the 3-minute incubation at 95°C was performed as part of the cycling parameters.
	The DNA polymerase was activated for more than 3 minutes. Ensure that the initial 95°C incubation was not longer than 3 minutes.
	Ensure the correct dilution of reference dye was used.
	Optimize the primer concentration.
	The $MgCl_2$ concentration is not optimal. The $MgCl_2$ concentration in the 1× Brilliant III SYBR Green QRT-PCR master mix is 2.5 mM. It is possible to add small amounts of concentrated $MgCl_2$ to the experimental reactions to increase the $MgCl_2$ concentration, if desired.
	The RNA template may be degraded. Ensure that the template RNA is stored properly (at –20°C or –80°C) and is not subjected to multiple freeze-thaw cycles. Check the quality of the RNA in the sample by gel electrophoresis or using an automated RNA population analysis system such as the Agilent 2100 Bioanalyzer.
	If the target RNA contains extensive secondary structure, increase the incubation temperature used during the first step of the RT-PCR program up to 55°C.
	For low-abundance targets or long amplicons, increase the duration of the cDNA synthesis step to 30-minutes while lowering the incubation temperature down to 42°C.
	Verify that all reagents and supplies are RNase-free.
	Where possible, increase the amount of template RNA. (Do not exceed the recommended amount of template.)
	Ensure the annealing/extension time is sufficient. Check the length of the amplicon and increase the annealing/extension time if necessary.
	Use a sufficient number of cycles in the PCR reaction.
	Gel analyze PCR product to determine if there was successful amplification.
In the dissociation plot, there is a large abundance of primer-dimer and nonspecific PCR products	Re-design primers.
	Optimize primer concentration by performing primer titration.
Increasing fluorescence in no-template control (NTC) reactions with cycling	Evaluate the dissociation profile. If Tm of the NTC peak is similar to the target peak, the reaction has been contaminated. Follow the procedures outlined in reference 3 to minimize contamination. If the Tm of the NTC peak is lower than the Tm of the target peak, primer-dimers are formed. Optimize primer concentration or re-design primer set.
Ct reported for NTC sample is less than the total number of cycles but the curve on the amplification plot is horizontal	Variation in fluorescence intensity. Review the amplification plot and, if appropriate, adjust the threshold accordingly.

REFERENCES

1. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology (N Y)* 11(9):1026-30.
2. Bustin, S. A. (2000) *Journal of Molecular Endocrinology* 25:169-193.
3. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

STRATAGENE

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BRILLIANT III ULTRA-FAST SYBR® GREEN QRT-PCR MASTER MIX

Catalog #600886, 600887

QUICK-REFERENCE PROTOCOL

Prior to setting up the reactions, thaw the 2× QRT-PCR master mix and store on ice. Following initial thawing of the master mix, the unused portion may be stored at 4°C for up to three months or returned to –20°C for long term storage.

SYBR Green I dye (present in the master mix) is light-sensitive; solutions containing the master mix should be protected from light whenever possible.

1. If using the reference dye, dilute the provided dye with nuclease-free PCR-grade H_2O. For the ABI StepOnePlus instrument or the ABI 7900HT Fast instrument, dilute the dye 1:50 (for a final concentration of 300 nM in the reactions). For the Stratagene Mx3000P or Mx3005P instrument or the ABI 7500 Fast instrument, dilute the dye 1:500 (for a final concentration of 30 nM in the reactions). Keep all solutions containing the reference dye protected from light.

2. Prepare the experimental reactions by adding the following components *in order*. Prepare a single reagent mixture for multiple reactions using multiples of each component listed below. *Keep the reagent mixture on ice*.

   ### Reagent Mixture

   Nuclease-free PCR-grade H_2O to bring the final volume to 20 µl (including experimental RNA)
   10 µl of 2× SYBR Green QRT-PCR master mix
   x µl of upstream primer (optimized concentration)
   x µl of downstream primer (optimized concentration)
   0.2 µl of 100 mM DTT
   0.3 µl of diluted reference dye from step 1 (optional)
   1 µl of RT/RNase block

3. Mix the reagents well without creating bubbles, then distribute the mixture to individual PCR reaction tubes. *Keep the reactions on ice*.

4. Add x µl of experimental RNA to each reaction.

5. Gently mix the reactions without creating bubbles, then centrifuge the reactions briefly.

6. Place the reactions in the instrument. Based on the instrument you are using, select the appropriate PCR program from the tables below. Set the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	12 seconds	60°C

ABI 7900HT Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	15 seconds	60°C

ABI StepOnePlus

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

QIAGEN Rotor-Gene Q

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	10 seconds	95°C
	10–20 seconds*	60°C

Roche LightCycler® 480

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5 seconds	95°C
1	10 seconds	60°C
	3 minutes	95°C

Stratagene Mx3000P and Mx3005P

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	5–20 seconds*	95°C
	20 seconds	60°C

7. For your specific instrument, follow the manufacturer's guidelines for generating dissociation curves.

* The exact duration needs to be optimized for each target. Challenging templates (e.g. GC-rich RNA or RNA with extensive secondary structure) generally require longer denaturation and annealing/extension times than low-complexity templates.