Brilliant III Ultra-Fast QRT-PCR Master Mix

Instruction Manual

Catalog #600884 (single kit)
#600885 (10-pack kit)
Revision F.0

For Research Use Only. Not for use in diagnostic procedures.

600884-12

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Brilliant III Ultra-Fast QRT-PCR Master Mix

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MATERIALS PROVIDED

Catalog #600884 (single kit), #600885 (10-pack kit)

Materials Provided	Quantity [a,b]
2× Brilliant III Ultra-Fast 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 one month or returned to –20°C for long term storage

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

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

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Revision F.0

INTRODUCTION

Quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The Brilliant III 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 master mix has been successfully used with fluorescent TaqMan® probes to amplify and detect a variety of high- and low-abundance RNA targets from experimental samples including total RNA, poly(A)$^+$ RNA, and synthetic RNA.

The kit includes the components necessary to carry out cDNA synthesis and PCR amplification in one tube and one buffer.* Brilliant kits support quantitative amplification and detection with multiplex capability. 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 Agilent 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 QRT-PCR 2× Master Mix

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

* Primers, probes and template are not included.

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.

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 master mix adaptable for many real-time QPCR platforms.

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, which reflects the change in fluorescence during cycling. This information can be used during PCR experiments to quantify initial copy number. Studies have shown that initial copy number can be quantified during real-time PCR analysis based on threshold cycle (Ct).[1] Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. The threshold cycle is 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 because threshold cycle is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents, small differences in reaction components, or accumulation of PCR inhibitors. Figure 1 shows an ABI StepOnePlus instrument amplification plot with Ct determination (top panel) and standard curve (bottom panel). In this experiment, the cyclophilin target was amplified and detected from total RNA using a TaqMan probe and the Brilliant III Ultra-Fast QRT-PCR master mix.

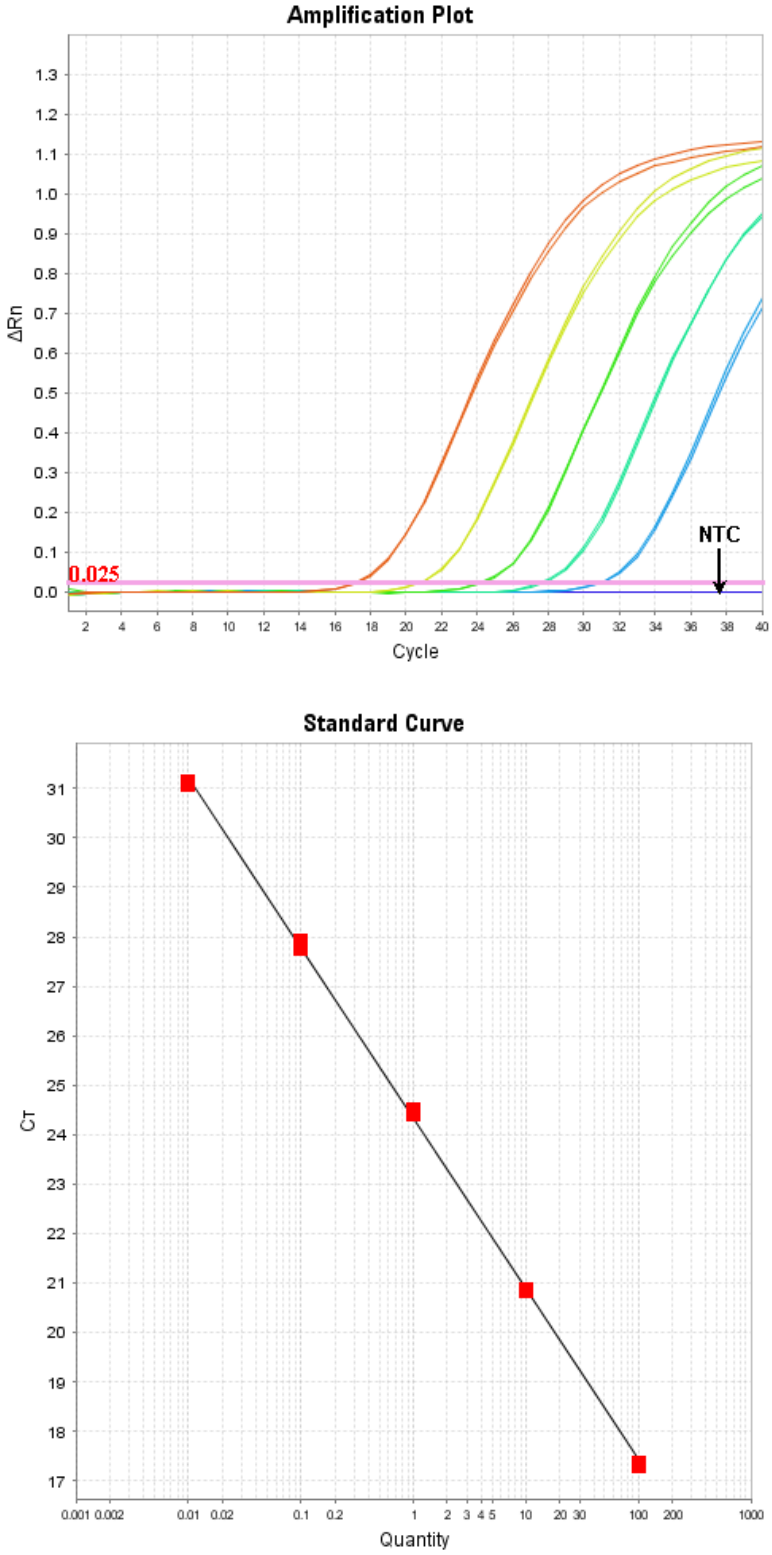

Figure 1 *Top panel*: StepOnePlus instrument amplification plot using a TaqMan® probe. A serial dilution of RNA template (ranging from 0.01–100 ng) was added to each reaction (set up in duplicate) . The fluorescence value used to determine Ct (the threshold line) is shown as a solid line. *Bottom panel*: Standard curve generated from amplification plot. An amplification efficiency of 93.2% and an R-squared value of 0.999 were obtained.

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 Agilent 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 optimally pure.

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

Agilent QPCR Human Reference Total RNA (Catalog #750500) is a high-quality control for quantitative PCR gene-expression analysis. Agilent 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.

Quantitative PCR 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 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 primer/probe systems. This eliminates the use of precious experimental RNA samples for assay optimization.

Probe Design

Probes should have a melting temperature that is 7–10°C higher than the annealing temperature of the primers. For additional considerations in designing TaqMan probes, refer to Primer Express® software from Applied Biosystems, the Primer3 program or a similar oligo design program.

Resuspend lyophilized custom TaqMan probes in buffer containing 5 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA (low TE buffer).

Optimal Concentrations for Experimental Probes and PCR Primers

Probes

The optimal concentration of the experimental TaqMan probe should be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 600 nM.

PCR Primers

The optimal concentration of the upstream and downstream PCR primers should also be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The primer concentration can be optimized by varying the concentration from 200 to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

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.

Treatment with Uracil-N-glycosylase (UNG) is NOT recommended for decontamination of single tube RT-PCR reactions since UNG would be active during the 50°C incubation necessary for reverse transcription.

Magnesium Chloride Concentration

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. The Brilliant III QRT-PCR master mix contains $MgCl_2$ at a concentration of 5.5 mM (in the 1× solution), which is suitable for most targets.

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 Agilent 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 and probes, prepare a single mixture of reaction components and then aliquot 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 QRT-PCR master mix. A 10-minute incubation for the first-strand synthesis reaction is sufficient for most targets

Multiplex RT-PCR

Multiplex RT-PCR is the amplification of more than one target in a single polymerase chain reaction.[4] The Brilliant III Ultra-Fast QRT-PCR master mix has been successfully used to amplify two targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase, reverse transcriptase or dNTPs.

In a typical multiplex RT-PCR reaction, one PCR primer pair primes the amplification of the target of interest and another PCR primer pair primes the amplification of an endogenous control. For accurate analysis, it is important to minimize competition between concurrent amplifications for common reagents. To minimize competition, the limiting primer concentrations need to be determined.[5] Consideration should also be given to optimization of the other reaction components. The number of fluorophores in each tube can influence the analysis. The use of a dark quencher, which emits heat instead of light, might enhance the quality of multiplex RT-PCR results by reducing the background light emission. The following PCR primer and probe design guidelines are useful for multiplex RT-PCR.

PCR Primer Considerations for Multiplex RT-PCR

- Design primer pairs with similar annealing temperatures for all targets to be amplified.
- To avoid duplex formation, analyze the sequences of primers and probes with primer analysis software.
- The limiting primer concentrations are the primer concentrations that result in the lowest fluorescence intensity without affecting the Ct. If the relative abundance of the two targets to be amplified is known, determine the limiting primer concentrations for the most abundant target. If the relative abundance of the two targets is unknown, determine the limiting primer concentrations for both targets. The limiting primer concentrations are determined by running serial dilutions of those forward and reverse primer concentrations optimized for one-probe detection systems, but maintaining a constant target concentration. A range of primer concentrations of 50–200 nM is recommended. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.[4]

TaqMan® Probe Considerations for Multiplex RT-PCR

- Label each TaqMan probe with a spectrally distinct fluorophore.
- Consider designing probes with dark quenchers.

Recommended Control Reactions

No Template Control (NTC)

We recommend performing no-template control reactions for each experimental sample to screen for amplicon contamination 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 3 for guidelines on the use of endogenous controls for QPCR.

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 one month, or return to –20°C for long term storage.*

It is prudent to set up a no-template control reaction to screen for amplicon contamination 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. See reference 3 for more information on endogenous controls.

Preparing the Reactions

1. If using the reference dye, dilute the provided dye using nuclease-free PCR-grade H_2O. **Keep all solutions containing the reference dye protected from light.**

   - 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 Agilent AriaMx, 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).

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× QRT-PCR master mix
   x µl of experimental probe (optimized concentration)
   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 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 to bring the final reaction volume to 20 µl. 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 Programs

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.

Note *For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system.*

Agilent AriaMx

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

Agilent 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

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	12 seconds	95°C
	15 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[c]	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	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	5–20 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

TROUBLESHOOTING

Observation	Suggestion
Little or no increase in fluorescence with cycling	The probe is not binding to its target efficiently because the annealing temperature is too high. Verify the calculated melting temperature using appropriate software.
	The target is highly GC-rich. Raise the denaturation temperature to 98°C or titrate DMSO into the reactions in 1% increments.
	The PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	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.
	For multiplex PCR, the $MgCl_2$ concentration may be increased, if desired, by adding a small amount of concentrated $MgCl_2$ (not provided in this kit) to the 1× experimental reaction at the time of set up.
	The probe has a nonfunctioning fluorophore. Verify that the fluorophore functions by performing a nuclease digestion to ensure it is unquenching as expected.
	Redesign the probe using Primer Express or other software. Design a probe that performs well in reactions containing 5.5 mM $MgCl_2$.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of the specific product by gel electrophoresis.
	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.)
	For multiplex PCR of more than two targets, reactions may need to be supplemented with additional polymerase and dNTPs (not provided).
Increasing fluorescence in no-template control reactions with cycling	The reaction has been contaminated. Follow the procedures outlined in reference 6 to minimize contamination.
Ct reported for the no-template control (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.

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ENDNOTES

ABI PRISM® is a registered trademark of Applied Biosystems.
LightCycler® is a registered trademark of Roche.
Primer Express® is a registered trademark of The Perkin-Elmer Corporation.
TaqMan® is a registered trademark of Roche Molecular Systems, Inc.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

BRILLIANT III ULTRA-FAST QRT-PCR MASTER MIX

Catalog #600884, #600885

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 one month, or returned to –20°C for long term storage.

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 an Agilent Mx 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× QRT-PCR master mix
   x µl of experimental probe (optimized concentration)
   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)
   1.0 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 to bring the final reaction volume to 20 µl.

5. Gently mix the reactions without creating bubbles, and 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.

Note *For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system.*

Agilent AriaMx

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

Agilent 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

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes	95°C
40	12 seconds	95°C
	15 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[c]	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	10 minutes	50°C
1	3 minutes[b]	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	5–20 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