Brilliant II QRT-PCR Master Mix Kit, 1-Step

Instruction Manual

Catalog #600809 (single kit)
#600818 (10-pack kit)
Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

600809-12

Agilent Technologies

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

United States and Canada

Agilent Technologies
Stratagene Products Division
11011 North Torrey Pines Road
La Jolla, CA 92037
Telephone (858) 373-6300
Order Toll Free (800) 424-5444
Technical Services (800) 894-1304
email techservices@agilent.com
World Wide Web www.genomics.agilent.com

Europe

Location	Telephone
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292

All Other Countries

Please contact your local distributor. A complete list of distributors is available at *www.genomics.agilent.com*.

Brilliant II QRT-PCR Master Mix Kit, 1-Step

CONTENTS

Brilliant II QRT-PCR Master Mix Kit, 1-Step

MATERIALS PROVIDED

Catalog #600809 (single kit), #600818 (10-pack kit)

Materials provided	Quantity [a,b]
2× Brilliant II QRT-PCR Master Mix	2 × 2.5 ml
RT/RNase Block Enzyme Mixture	400 µl
Reference dye [c], 1 mM	100 µl

[a] Sufficient PCR reagents are provided for four hundred, 25-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: Upon receipt, store all components at –20°C. Store the 2× master mix at 4°C after thawing. Once thawed, full activity is guaranteed for 6 months.

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

NOTICES TO PURCHASER

Notice to Purchaser: Limited License

Practice of the patented 5' Nuclease Process requires a license from Applied Biosystems. The purchase of this product includes an immunity from suit under patents specified in the product insert to use only the amount purchased for the purchaser's own internal research when used with the separate purchase of Licensed Probe. No other patent rights are conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Revision C

INTRODUCTION

Quantitative PCR is a powerful tool for gene expression analysis. Many fluorescent chemistries are used to detect and quantitate gene transcripts. The use of fluorescent probe technologies reduces the risk of sample contamination while maintaining convenience, speed, and high-throughput screening capabilities. The Brilliant II QRT-PCR Master Mix Kit, 1-Step can be used with both hairpin and linear fluorescent probe technologies to perform absolute or relative quantitation of gene expression. The single-step master mix format is ideal for most high-throughput QPCR applications where it is not necessary to archive cDNA.

The Brilliant II QRT-PCR master mix 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 and show consistent high performance with various fluorescent detection systems, including molecular beacons and TaqMan® probes. The Brilliant II QRT-PCR master mix kit has been successfully used 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 Brilliant II QRT-PCR master mix has been optimized for maximum performance on the Stratagene Mx3000P and Mx3005P real-time PCR systems and the Stratagene Mx4000 multiplex quantitative PCR system, as well as on the ABI 7900HT real-time PCR instrument.

Features of Kit Components

RT/RNase Block Enzyme Mixture

The reverse transcriptase (RT) provided in the kit is a Moloney-based RT specifically formulated for Stratagene Brilliant II kits. This RT performs optimally at a reaction temperature of 50°C when used in 1-step QRT-PCR with the Brilliant II 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.

* Primers and template are not included.

Brilliant II QRT-PCR 2× Master Mix

The 2× master mix contains an optimized RT-PCR buffer, $MgCl_2$, nucleotides (GAUC), stabilizers, and SureStart *Taq* DNA polymerase. SureStart *Taq* DNA polymerase is a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR performance by decreasing background and increasing amplification of desired products. Using SureStart *Taq*, hot start is easily incorporated into PCR protocols already optimized with *Taq* DNA polymerase, with little or no modification of cycling parameters or reaction conditions.

Reference dye

A passive reference dye (an optional reaction component) is provided in a third tube. The passive reference dye (with excitation and emission wavelengths of 584 nm and 612 nm, respectively) is provided 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 II QRT-PCR master mix kit adaptable for many real-time QPCR platforms (see *Reference Dye* in *Preprotocol Considerations* for more information).

Molecular Beacons Probes

Molecular beacons are hairpin-shaped fluorescent hybridization probes that can be used to monitor the accumulation of specific product during or after PCR.[1–5] Molecular beacons have a fluorophore and a quencher molecule at opposite ends of an oligonucleotide (see Figure 1). The ends of the oligonucleotide are designed to be complementary to each other. When the unhybridized probe is in solution, it adopts a hairpin structure that brings the fluorophore and quencher sufficiently close to each other to allow efficient quenching of the fluorophore. If, however, the molecular beacon is bound to its complementary target, the fluorophore and quencher are far enough apart that the fluorophore cannot be quenched and the molecular beacon fluoresces. As PCR proceeds, product accumulates and the molecular beacon fluoresces at a wavelength characteristic of the particular fluorophore used. The amount of fluorescence at any given cycle depends on the amount of specific product present at that time.

TaqMan® Probes (Hydrolysis Probes)

TaqMan probes are linear.[6, 7] The fluorophore is usually at the 5´ end of the probe, and the quencher is either internal or is at the 3´ end (see Figure 2). As long as the probe is intact, regardless of whether it is hybridized with the target or free in solution, no fluorescence is observed from the fluorophore. During the combined annealing-extension step of PCR, the primers and the TaqMan probe hybridize with the target. The DNA polymerase displaces the TaqMan probe by 3 or 4 nucleotides, and the 5´-nuclease activity of the DNA polymerase separates the fluorophore from the quencher. Because of this mechanism of action, these probes are also referred to as hydrolysis probes. Fluorescence can be detected during each PCR cycle, and fluorescence accumulates during the course of PCR.

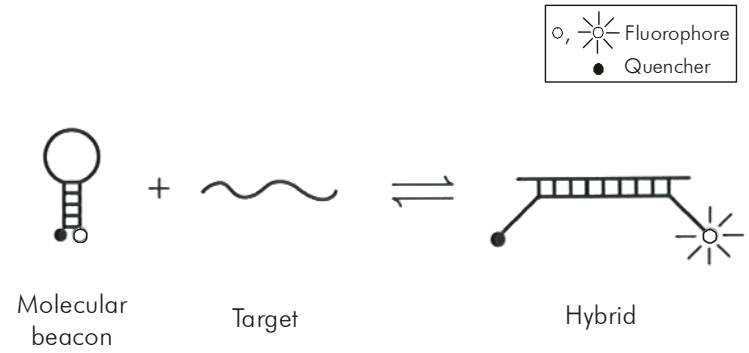


FIGURE 1 The molecular beacon binds to a complementary target and fluoresces.

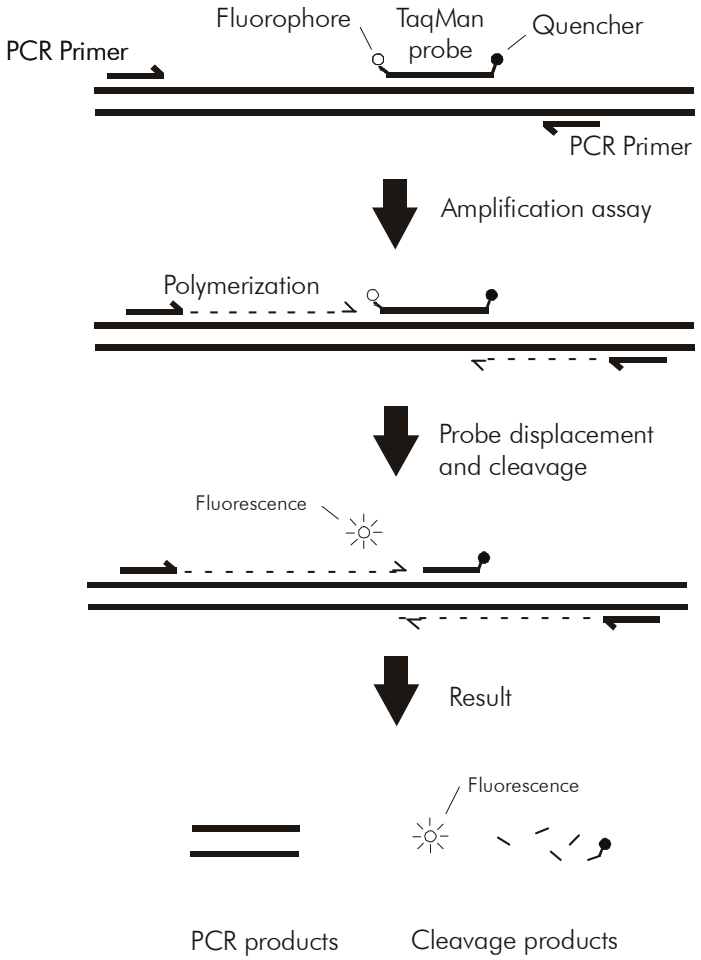
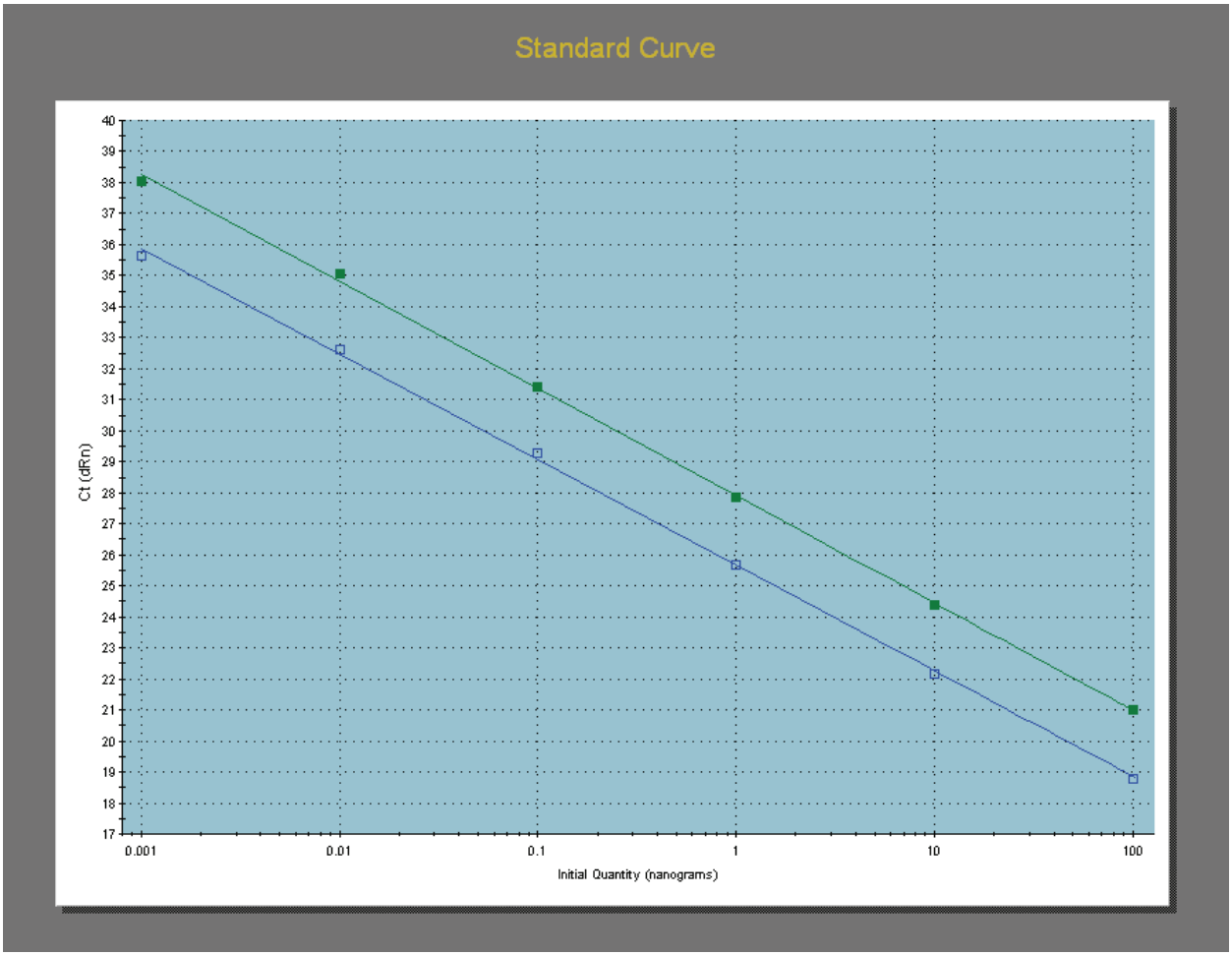


FIGURE 2 TaqMan probe fluoresces when the 5′-nuclease activity of the DNA polymerase separates the fluorophore from quencher.

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 real-time PCR experiments to quantitate initial copy number based on the threshold cycle (Ct).[6] 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.[6] 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 cycling conditions.

Ct values determined for a set of standard wells, containing known amounts of the target, may be plotted to generate a standard curve that can be used to relate Ct values to initial copy number for unknown samples. Figure 3 shows Mx3000P instrument standard curve plots for the GAPDH gene and the cyclophilin gene from a multiplex QRT-PCR experiment using TaqMan probes. In this experiment, serial dilutions of total RNA were reverse-transcribed and amplified with fluorescence detected at each cycle. The table shows the R^2 values and PCR efficiencies calculated by the Mx3000P instrument from the standard curve plots. The R^2 value (always between 0 and 1) is an indication of the quality of the fit of the standard curve to the standard data points plotted, with values closer to 1 indicating a better fit of the data to the line. The slope of the standard curve is directly related to the average efficiency of amplification throughout the cycling program and may be used to calculate the PCR efficiency for a given template in a given experiment. A reaction with 100% efficiency will produce a slope of –3.322.



Target	Symbol	R^2 Value	Slope	Efficiency (%)
Cyclophilin	■ (closed squares)	0.999	-3.455	94.7
GAPDH	□ (open squares)	0.999	-3.404	96.7

Figure 3 Mx3000P quantitative PCR instrument standard curve plots using TaqMan probes for GAPDH (open squares) or cyclophilin (closed squares) in multiplex single-tube RT-PCR reactions. The table below the standard curve plot shows the R^2 value, standard curve slope and amplification efficiency for each of the targets.

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 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 RT/RNase block enzyme mixture 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 QPCR Human Reference Total RNA (Catalog #750500) is a high-quality control for quantitative PCR gene-expression analysis. Stratagene 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® oligo design software from Applied Biosystems.

Resuspend lyophilized custom molecular beacon or 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 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.

A) Molecular Beacons

The molecular beacon concentration can be optimized by varying the final concentration from 200 to 500 nM in increments of 100 nM.

B) TaqMan® Probes

The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 500 nM in increments of 100 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 for use with molecular beacons can be optimized by varying the concentration from 200 to 600 nM. The primer concentration for use with TaqMan probes can be optimized by varying the concentration from 100 to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

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 optional when using the Mx4000, Mx3000P or Mx3005P system, with other instruments (including the ABI 7900HT and ABI PRISM® 7700) 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 you are using a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If you are using the ABI 7900HT real-time PCR instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

Magnesium Chloride Concentration

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

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 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.

* 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.

Mixing and Pipetting Enzymes

Solutions that contain enzymes (including reverse transcriptase and SureStart *Taq* 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, which contains 50% glycerol, can lead to pipetting errors.

Temperature and Duration of cDNA Synthesis Reaction

For cDNA synthesis, we recommend a 50°C incubation for most targets using the Brilliant II QRT-PCR master mix kit. However, incubation up to 55°C can be employed to reduce secondary structures or to improve specificity. A 30-minute incubation for the first-strand synthesis reaction is sufficient for most targets. Rare RNA sequences or long amplicons may benefit from an extended incubation time (up to 60 minutes) at a lower temperature (42°C).

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.

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 enzyme mixture 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 8 for guidelines on the use of endogenous controls for QPCR.

Endpoint vs. Real-Time Measurements

Fluorescence may be detected either at the endpoint of cycling or in real-time using a real-time spectrofluorometric thermal cycler. Real-time experiments are typically performed on an instrument capable of detecting fluorescence from samples during each cycle of a PCR protocol. For endpoint analysis, PCR reactions can be run on any thermal cycler and can then be analyzed with a fluorescence plate reader that has been designed to accommodate PCR tubes and that is optimized for the detection of PCR reactions that include fluorescent probes. If using a fluorescence plate reader, it is recommended that readings be taken both before and after PCR for comparison.

Data Acquisition with a Spectrofluorometric Thermal Cycler

Acquisition of real-time data generated by fluorogenic probes should be performed as recommended by the instrument's manufacturer.

When developing an assay, it is necessary to decide whether to use a 2-step or a 3-step PCR protocol. We recommend a 2-step protocol for the Brilliant II QRT-PCR master mix kit. In a 2-step cycling protocol, fluorescence data are collected during the combined annealing/extension step. When using a 3-step protocol, it is prudent to collect fluorescence data at both the annealing step and the extension step of the PCR reaction.

Multiplex RT-PCR

Multiplex RT-PCR is the amplification of more than one target in a single polymerase chain reaction.[9] The Brilliant II QRT-PCR master mix kit 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.[10] 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.[10]

Probe Considerations for Multiplex RT-PCR

A) Molecular Beacons

- Label each molecular beacon with a spectrally distinct fluorophore.[11]
- Consider designing probes with dark quenchers.
- Design molecular beacons for different targets to have different stem sequences.

B) TaqMan® Probes

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

PROTOCOL

Notes *Following initial thawing of the master mix, store the unused portion at 4°C. Multiple freeze-thaw cycles should be avoided.*

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 8.

Preparing the Reactions

1. If the reference dye will be included in the reaction, (optional), dilute the dye solution provided **1:500 (for the Mx3000P, Mx3005P, and Mx4000 instruments)** or **1:50 (for the ABI 7900HT real-time PCR instrument)** using nuclease-free PCR-grade H_2O. For other instruments, use the guidelines in the *Reference Dye* section under *Preprotocol Considerations*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the Mx3000P, Mx3005P, and Mx4000 instruments and 300 nM for the ABI 7900HT instrument. **Keep all solutions containing the reference dye protected from light.**

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× Brilliant II QRT-PCR master mix and store on ice. Mix the solution well by gentle inversion prior to pipetting.

3. Prepare the experimental reactions by combining the following components *in order*. Prepare a single reagent mixture for duplicate experimental reactions and duplicate no-template controls (plus at least one reaction volume excess), using multiples of each component listed below.

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental RNA)
12.5 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)
0.375 µl of the **diluted** reference dye (optional)
1.0 µl of RT/RNase block enzyme mixture

Note *A total reaction volume of 50µl may also be used.*

4. Gently mix the reagents without creating bubbles (do not vortex), then distribute the mixture to individual PCR reaction tubes.

5. 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 1 pg–400 ng of total RNA or 0.1 pg–1 ng of mRNA.

6. Gently mix the reactions without creating bubbles (do not vortex).

   Note *Bubbles interfere with fluorescence detection.*

7. Centrifuge the reactions briefly.

RT-PCR Cycling Programs

8. Place the reactions in the QPCR instrument and run the appropriate RT-PCR program using the guidelines in the tables below. The 2-step cycling protocol is preferred for most primer/template systems.

Two-Step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40	15 seconds	95°C
	1 minute[b]	60°C

[a] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[b] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

Alternative Protocol with Three-Step Cycling

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1 minute[b]	50–60°C[c]
	30 seconds	72°C

[a] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[b] Set the temperature cycler to detect and report fluorescence during the annealing and extension step of each cycle.

[c] Choose an appropriate annealing temperature for the primer set used.

TROUBLESHOOTING: TAQMAN® PROBES

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 probe is not binding to its target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	Design a probe that is compatible with 5.5 mM $MgCl_2$.
	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 digesting the probe (100 nM probe in 25 µl 1× buffer with 10 U DNase or S1 nuclease) at room temperature for 30 minutes to confirm an increase in fluorescence following digestion.
	Redesign the probe.
	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 to up to 55°C.
	For low-abundance targets or long amplicons, increase the duration of the cDNA synthesis step to 60-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 12 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.

TROUBLESHOOTING: MOLECULAR BEACONS

Observation	Suggestion
Little or no increase in fluorescence with cycling	The molecular beacon is not binding to its target efficiently because the loop portion is not completely complementary. Perform a melting curve analysis to determine if the probe binds to a perfectly complementary target.
	The molecular beacon is not binding to its target efficiently because the annealing temperature is too high. Perform a melting curve analysis to determine the optimal annealing temperature.
	The molecular beacon is not binding to its target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	Design the molecular beacon with a stem that is compatible with 5.5 mM $MgCl_2$.
	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 molecular beacon has a nonfunctioning fluorophore. Verify that the fluorophore functions by detecting an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. If there is no increase in fluorescence, resynthesize the molecular beacon.
	Resynthesize the molecular beacon using a different fluorophore.
	Redesign the molecular beacon.
	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 60-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 12 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.

REFERENCES

1. Kostrikis, L. G., Tyagi, S., Mhlanga, M. M., Ho, D. D. and Kramer, F. R. (1998) *Science* 279(5354):1228-9.
2. Padmabandu, G., Grismer, L. and Mueller, R. (2000) *Strategies* 13(3):88-92.
3. Piatek, A. S., Tyagi, S., Pol, A. C., Telenti, A., Miller, L. P. et al. (1998) *Nat Biotechnol* 16(4):359-63.
4. Tyagi, S., Bratu, D. P. and Kramer, F. R. (1998) *Nat Biotechnol* 16(1):49-53.
5. Tyagi, S. and Kramer, F. R. (1996) *Nat Biotechnol* 14(3):303-8.
6. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology* (N Y) 11(9):1026-30.
7. Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. (1991) *Proc Natl Acad Sci U S A* 88(16):7276-80.
8. Bustin, S. A. (2000) *Journal of Molecular Endocrinology* 25:169-193.
9. Edwards, M. and Gibbs, R. (1995). Multiplex PCR. In *PCR Primer: A Laboratory Manual*,C. W. Dieffenbach and G. S. Dveksler (Eds.), pp. 157-171. Cold Spring Harbor Laboratory Press, Plainview, NY.
10. McBride, L., Livak, K., Lucero, M., Goodsaid, F., Carlson, D. et al. (1998). Quantitative PCR Technology. In *Gene Quantification*,F. Ferré (Ed.), pp. 97-110. Birkhauser Boston Press, Boston.
11. Marras, S. A., Kramer, F. R. and Tyagi, S. (1999) *Genet Anal* 14(5-6):151-6.
12. 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

An **Agilent Technologies** Division

BRILLIANT II QRT-PCR MASTER MIX, 1-STEP

Catalog #600809, #600818

QUICK-REFERENCE PROTOCOL

Note *This protocol has been optimized for the Stratagene Mx3000P, Mx3005P, and Mx4000 instruments and the ABI 7900HT instrument. The protocol may be adapted for use with most other instruments by changing the reference dye dilution according to the guidelines in the manual and following the instrument manufacturer's recommendations for RT-PCR cycling programs.*

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (Mx3000P, Mx3005P, or Mx4000 instrument) or 1:50 (ABI 7900HT instrument). Keep all solutions containing the reference dye protected from light.

   Note *If using a system other than the Mx4000, Mx3000P or Mx3005P instruments, the use of the reference dye may be required for optimal results.*

2. Thaw the 2× QRT-PCR master mix and store on ice. Following initial thawing of the master mix, store the unused portion at 4°C.

   Note *Multiple freeze-thaw cycles should be avoided.*

3. 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.

   ### Reagent Mixture

   Nuclease-free PCR-grade H_2O to bring the final volume to 25 µl (including experimental RNA)
   - 12.5 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)
   - 0.375 µl of diluted reference dye from step 1 (optional)
   - 1.0 µl of RT/RNase block mixture

   Note *A total reaction volume of 50µl may also be used.*

4. Gently mix the reagents without creating bubbles (**do not vortex**), then distribute the mixture to individual PCR reaction tubes.

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

6. Gently mix the reactions without creating bubbles (**do not vortex**).

7. Centrifuge the reactions briefly.

8. Place the reactions in the instrument and run the appropriate PCR program below.

Two-Step Cycling Protocol[a]

Cycles	Duration of cycle	Temperature
1	30 minutes	50°C
1	10 minutes[b]	95°C
40	15 seconds	95°C
	1 minute[c]	60°C

[a] A protocol for three-step cycling is provided in the *Protocol* section.

[b] Initial 10 minute incubation is required to fully activate the DNA polymerase.

[c] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.