Brilliant II QRT-PCR Core Reagent Kit, 1-Step

Instruction Manual

Catalog #600810 (single kit)
#600819 (10-pack kit)
Revision C.0

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600810-12

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MATERIALS PROVIDED

Catalog #600810 (single kit), #600819 (10-pack kit)

Materials provided	Quantity [a,b]
Reverse Transcriptase	400 µl
10× Core RT-PCR buffer	1.7 ml
SureStart *Taq* DNA polymerase (5 U/µl)	500 U
50 mM $MgCl_2$	1500 µl
20 mM dNTP mix (5 mM each of dATP, dTTP, dGTP, and dCTP)	400 µl
Reference dye[c] (1 mM)	100 µl

[a] Sufficient PCR reagents are provided for four hundred 25-µl 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.

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

ADDITIONAL MATERIALS REQUIRED

Nuclease-free PCR-grade water
Thermal cycler and fluorescence reader
or
Spectrofluorometric thermal cycler

Note *A heated lid for the thermal cycler is strongly recommended because it allows optimal detection of the fluorescence. An overlay of mineral oil slightly attenuates the fluorescence.*

Revision C.0

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INTRODUCTION

Quantitative PCR is a powerful tool for gene expression analysis. Many fluorescence 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 Core Reagent Kit, 1-Step can be used with both hairpin and linear fluorescent probe technologies to perform absolute or relative quantitation of gene expression. The 1-step format is ideal for most high-throughput QPCR applications where it is not necessary to archive cDNA.

The Brilliant II QRT-PCR core reagent 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 1-step core reagent 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)$^+$, and synthetic RNA.

The Brilliant II QRT-PCR core reagent kit has been optimized for maximum performance on Agilent Mx3000P, Mx3005P, and Mx4000 instruments, as well as on the ABI 7900HT real-time PCR instrument.

Features of Kit Components

The Brilliant II QRT-PCR core reagent kit includes SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability. SureStart *Taq* DNA polymerase improves PCR amplification reactions 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.

The reverse transcriptase (RT) provided in the kit is a Moloney-based RT specifically formulated for Agilent 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 core reagents. 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.

* Primers and template are not included.

Molecular Beacons

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. 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 (see Figure 1). 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 will depend 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. 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 annealing/extension step of PCR, the primers and the TaqMan probe hybridize with the target (see Figure 2). 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. Fluorescence can be detected during each PCR cycle, and fluorescence accumulates during the course of PCR.

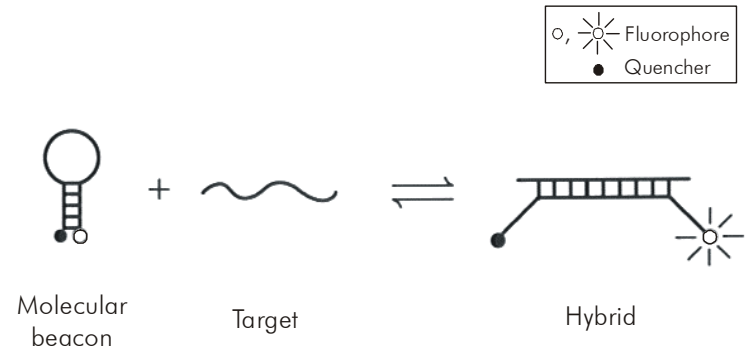

FIGURE 1 The molecular beacon binds to a complementary target and fluoresces.

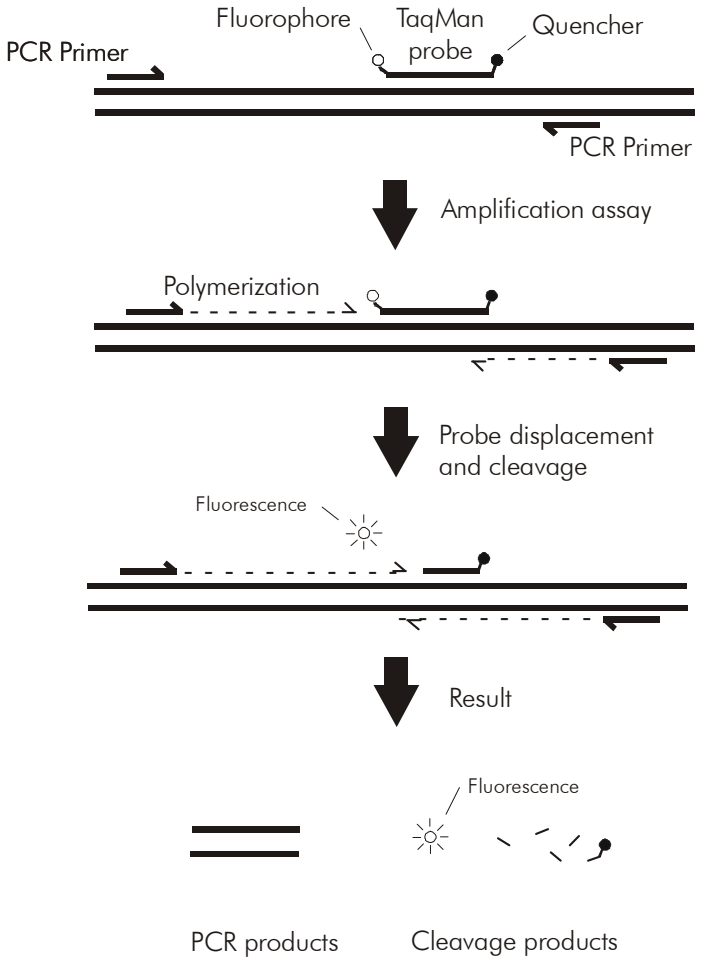

FIGURE 2 TaqMan probe fluoresces when the 5′-nuclease activity of the DNA polymerase separates the fluorophore from the quencher.

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. Use of an RNase inhibitor (added to the reaction prior to the addition of the RNA target) such as RNase Block ribonuclease inhibitor (Agilent catalog #300151) is recommended when isolating RNA from samples high in RNase activity.

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

The 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.

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 using an Agilent Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If using the ABI 7900HT 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.

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).

* 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.

Optimal Concentrations for Probes and 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. The conditions that produce the lowest amounts of primer dimer and nonspecific product are recommended.

Magnesium Chloride

Generally, a magnesium chloride concentration of 5 mM yields good results for most targets, but we recommend optimizing the $MgCl_2$ concentration for each experimental molecular beacon or TaqMan probe. The optimal concentration is the concentration that results in the lowest Ct and highest fluorescence for a given target concentration. The $MgCl_2$ concentration can be optimized by starting with a concentration of 5 mM and then varying the concentration in increments of 0.5 mM.

Reverse Transcriptase

In a 25-µl QRT-PCR reaction, we recommend using 1 µl of reverse transcriptase (RT). However, for some primer/probe systems, performance may be improved by optimizing the amount of RT. To determine the optimal amount, vary the volume of RT added to the reaction from 0.1µl to 4 µl.

Endpoint vs. Real-Time Measurements

The fluorescence of the probe can be monitored either when cycling is complete (endpoint analysis) or as the reaction is occurring (real-time analysis). 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. Real-time experiments are typically performed on an instrument capable of detecting fluorescence from samples during each cycle of a PCR protocol.

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, but a 3-step protocol may be helpful when using primers with low melting temperatures. 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. For subsequent experiments, the plateau resulting in low Ct values for the samples containing target and high Ct values (or "no Ct" values) for the controls containing no target should be chosen for analysis. For longer amplicons, fluorescence measurements taken during the extension step generally yield more useful data.

Recommended Controls

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

Consider performing no-RT control reactions for each experimental sample by omitting reverse transcriptase from the reaction. The no-RT control is expected to generate no signal if the primers are specific for the cDNA and do not amplify genomic DNA (see *Troubleshooting*).

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.

PCR Control

Consider performing RT-PCR with a control DNA template as a control for the PCR component of RT-PCR.

Temperature and Duration of cDNA Synthesis Reaction

For cDNA synthesis, a 50°C incubation is recommended for most targets when using the Brilliant II QRT-PCR core reagent 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).

Multiplex RT-PCR

Multiplex RT-PCR is the amplification of more than one target in a single polymerase chain reaction.[9] In a typical multiplex RT-PCR, 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 might enhance the quality of multiplex RT-PCR results. The following 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 2-fold 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.[9]

Magnesium Chloride Concentration in Multiplex RT-PCR

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. Consider performing a titration to optimize the magnesium chloride concentration for multiplex RT-PCR.

DNA Polymerase and dNTP Concentrations in Multiplex RT-PCR

Multiplex RT-PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.[11] The Brilliant II QRT-PCR core reagent kit has been successfully used to amplify two targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase or dNTPs. If more than two targets are amplified and detected in a single reaction tube, optimal polymerase and dNTP concentrations may need to be determined empirically.

Probe Considerations for Multiplex RT-PCR

A) Molecular Beacon

- Label each molecular beacon with its own spectrally distinct fluorophore.[12]
- Design molecular beacons for different targets to have different stem sequences.

B) TaqMan Probes

Label each TaqMan probe with a spectrally distinct fluorophore.

General Notes

Preventing Cross-Contamination

Take precautions to minimize the potential for carryover of nucleic acids (RNA and DNA) 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.

Preparing a Master Mix for Multiple Samples

If running multiple samples, a master mix of reaction components can be prepared by combining the desired multiple of each component. Individual samples can then be prepared by aliquoting the master mix into individual tubes using a fresh pipet tip for each addition. Using a master mix facilitates accurate dispensing of reagents, minimizes loss of reagents during pipetting, and makes repeated dispensing of each reagent unnecessary, all of which help minimize sample-to-sample variation.

Mixing and Pipetting Enzymes

Enzymes (including reverse transcriptase and SureStart *Taq* DNA polymerase) should be mixed by gentle vortexing without generating bubbles. Pipet the enzymes carefully and slowly; otherwise, the viscosity of the 50% glycerol in the buffer can lead to pipetting errors.

PROTOCOL

Preparing the Reactions

Notes *Gently mix and spin each component in a microcentrifuge before use.*

Prepare all reactions on ice.

The preferred method for preparing reactions is to make master mixes by combining the desired multiple of each component listed in step 2. Individual samples can then be prepared by pipetting the master mixes into PCR tubes or plates and then adding the RNA template. Use PCR tubes or plates that are recommended by the manufacturer of the spectrofluorometric thermal cycler.

It is prudent to set up a no-template control reaction to screen for contamination of reagents or false amplification.

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 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. Prepare the reactions by adding the following components *in order*:

Reagent Mixture

Nuclease-free PCR-grade water to adjust the final volume to 25µl (including experimental RNA)
2.5 µl of 10× core RT-PCR buffer
x µl of 50 mM $MgCl_2$ (optimize for each primer–probe system starting at 5 mM)
x µl of upstream primer
x µl of downstream primer
x µl of experimental probe
1.0 µl of 20 mM dNTP mix
1.0 µl of reverse transcriptase (or use optimized volume)
0.375 µl of diluted reference dye (optional)
0.25 µl of SureStart *Taq* DNA polymerase

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

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

4. Spin the reactions in a microcentrifuge and aliquot into individual PCR reaction tubes.

5. Add *x* µl of RNA (the quantity, typically <200 ng, depends on message abundance) to each reaction.

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 Program

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. For primers with low melting temperatures, consider using the alternative 3-step cycling protocol.

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 combined 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

MOLECULAR BEACONS

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The molecular beacon is not binding to the 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 the 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 the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	The molecular beacon is not binding to the target efficiently because the magnesium concentration is too low. Perform a magnesium titration to optimize the concentration.
	Not enough or no specific product is formed during PCR. Verify product formation by gel electrophoresis.
	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 using a different fluorophore.
	Redesign the molecular beacon.
	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.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 13 to minimize contamination.
There is an increase in fluorescence in the no-RT control reactions	The primers and/or molecular beacon are not specific for cDNA, and the signal is produced from contaminating genomic DNA. Redesign the primers or molecular beacon to recognize only product amplified from cDNA by positioning the molecular beacon to span a splice site.
	Treat RNA with DNase prior to RT to digest contaminating genomic DNA. Confirm the complete digestion of DNA by running another minus-RT control.
For a given primer–molecular beacon set, DNA reactions yield signal but RNA reactions do not	RNA is degraded. Replace RNA and use RNase inhibitor in the reactions.
	A component in the RNA sample is inhibiting the reaction. Purify the target RNA samples.
	Reverse transcriptase is nonfunctional. Ensure that the reverse transcriptase is not heat-deactivated. Do not use RT at temperatures higher than 55°C.
Ct reported for the no-target control sample (NTC) in experimental report 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.

TaqMan® Probes

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The probe is not binding to the target efficiently because the annealing temperature is too high. Verify the melting temperature using appropriate software.
	The probe is not binding to the target efficiently because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	The probe is not binding to the target efficiently because the magnesium concentration is too low. Perform a magnesium titration to optimize the concentration.
	Not enough or no specific product is formed during PCR. Verify product formation by gel electrophoresis.
	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.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 13 to minimize contamination.
There is an increase in fluorescence in the no-RT control reactions	The primers and/or probe are not specific for cDNA, and the signal is produced from contaminating genomic DNA. Redesign the primers or probe to recognize only product amplified from cDNA by positioning the probe to span a splice site.
	Treat RNA with DNase prior to RT to digest contaminating genomic DNA. Confirm the complete digestion of DNA by running another minus-RT control.
For a given primer–probe set, DNA reactions yield signal but RNA reactions do not	RNA is degraded. Replace RNA and use RNase inhibitor in the reactions.
	A component in the RNA sample is inhibiting the reaction. Purify the target RNA samples.
	Reverse transcriptase is nonfunctional. Ensure that reverse transcriptase is not heat-deactivated. Do not use reverse transcriptase at temperatures higher than 55°C.
Ct reported for the no-target control sample (NTC) in experimental report 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.

APPENDIX: REAL-TIME RT-PCR METHODS FOR QUANTITATING RNA

Relative Quantitation Using the Standard Curve Method

Note *Generate a standard curve for each target.*

1. Generate standards by preparing 10-fold serial dilutions of an RNA sample of known quantity, starting with 100 ng.

2. Prepare RT-PCR reactions using primers and molecular beacon or TaqMan probe for the experimental target and for an endogenous control target.

3. Place the reactions in a spectrofluorometric thermal cycler and run the two-step cycling program given in the *Protocol*.

4. Calculate the Ct for each dilution of the standard and for the unknown experimental and endogenous control samples.

5. Generate standard curves for each target by plotting the Ct of each standard dilution against the logarithm of the initial amount of RNA. Calculate the regression line for each target.

6. Calculate the logarithms of the initial concentrations of both the experimental target and the endogenous control target using the following formula:

   $$\log(\textit{initial target concentration}) = (Ct - b)/a$$

   where Ct = threshold cycle, b = y-intercept of the standard curve, and a = slope of the standard curve.

7. Divide the amount of experimental RNA by the amount of endogenous control to normalize the amount of experimental RNA.

8. Designate one of the samples as a calibrator. An untreated control or the sample with the lowest level of expression is an appropriate calibrator. Fold differences in expression can be determined by dividing the normalized amount of experimental RNA (from step 7) by the normalized amount of the calibrator.

Absolute Quantitation

The Brilliant II QRT-PCR core reagent kit can be used for absolute gene quantitation with both molecular beacons and TaqMan probes. See reference 14 for methods.

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ENDNOTES

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 II QRT-PCR CORE REAGENT KIT, 1-STEP

Catalog #600810 and #600819

QUICK-REFERENCE PROTOCOL

Prior the experiment, it is prudent to carefully optimize experimental conditions and to include controls at every stage. See *Preprotocol Considerations* for details.

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 or 1:50 depending on the detection instrument used. See guidelines in *Reference Dye* in the *Preprotocol Considerations* section.

   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. Prepare the 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 water to adjust the final volume to 25 µl (including RNA)
   - 2.5 µl of 10× core RT-PCR buffer
   - x µl of 50 mM $MgCl_2$ (optimize for each primer–probe system starting at 5 mM)
   - x µl of upstream primer
   - x µl of downstream primer
   - x µl of experimental probe
   - 1.0 µl of 20 mM dNTP mix
   - 1.0 µl of reverse transcriptase (or use optimized volume)
   - 0.375 µl of diluted reference dye (optional)
   - 0.25 µl of SureStart *Taq* DNA polymerase

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

3. Gently mix the reactions without creating bubbles (**bubbles interfere with fluorescence detection; do not vortex**).

4. Spin the reactions in a microcentrifuge and aliquot into individual PCR reaction tubes.

5. Add x µl of RNA (the quantity, typically <200 ng, depends on message abundance) to each reaction.

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

7. Centrifuge the reactions briefly.

8. Place the reactions in a thermal cycler and run the 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 three-step cycling protocol 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.