Brilliant II QRT-PCR, AffinityScript Two-Step Master Mix

Instruction Manual

Catalog #600827

Revision D.0

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600827-12

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Brilliant II QRT-PCR, AffinityScript Two-Step Master Mix

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Brilliant II QRT-PCR, AffinityScript Two-Step Master Mix

MATERIALS PROVIDED

Catalog #600827

Materials Provided	Concentration	Quantity
AffinityScript QPCR cDNA Synthesis Kit[a]		
cDNA Synthesis Master Mix	2×	500 µl
AffinityScript RT/ RNase Block Enzyme Mixture	—	50 µl
Oligo(dT) primer	100 ng/µl	20 µg
Random primers	100 ng/µl	20 µg
RNase-free H_2O	—	1.2 ml
Brilliant II QPCR Master Mix[b]		
Brilliant II QPCR Master Mix	2×	2 × 2.5 ml
Reference dye[c]	1 mM	100 µl

[a] Sufficient reagents are provided for fifty, 20-µl cDNA synthesis reactions.

[b] Sufficient reagents are provided for four hundred, 25-µl QPCR reactions.

[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× Brilliant II QPCR 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

Revision D.0

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INTRODUCTION

Real-time quantitative reverse transcription PCR (QRT-PCR) is a powerful tool for gene expression analysis. The Brilliant II QRT-PCR, AffinityScript Two-Step Master Mix provides a streamlined, fully optimized system for two-step QRT-PCR applications. A two-step RT-PCR format is useful for amplifying multiple targets from a single cDNA source and for maintaining archival cDNA. The kit is composed of two modules, both of which are optimized for streamlined QRT-PCR experiments. First, RNA is reverse-transcribed using the AffinityScript QPCR cDNA Synthesis Kit with a master mix format and fast protocol with a 15-minute cDNA synthesis step and an overall protocol time of just 25 minutes. Second, the cDNA of interest is quantified using the Brilliant II QPCR Master Mix.

Reverse Transcription with the AffinityScript QPCR cDNA Synthesis Kit

The AffinityScript QPCR cDNA synthesis kit provides a comprehensive set of reagents necessary to generate high-quality, QPCR-ready cDNA templates up to 12 kb in length from either poly(A)$^+$ mRNA or total RNA. Key to the performance of the kit is the QPCR-grade AffinityScript reverse transcriptase (RT) enzyme and the corresponding QPCR-optimized RT buffer. The first-strand synthesis step is completed using a convenient master mix with few pipetting steps, saving you time and ensuring experiment-to-experiment reproducibility. Each reaction accommodates a range of RNA amounts from fg to µg.

cDNA Synthesis Master Mix

The cDNA synthesis master mix contains a buffer that is specifically optimized for QRT-PCR performance, allowing a fast protocol and reducing variability in Ct measurements between reactions. In addition to the optimized buffer, the master mix contains $MgCl_2$, and dNTPs.

AffinityScript RT/RNase Block Enzyme Mixture

The kit includes our QPCR-grade AffinityScript Multiple Temperature Reverse Transcriptase, a genetically engineered version of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT), which is highly thermostable, allowing you to reverse transcribe at your preferred reaction temperature. AffinityScript RT is provided, in combination with RNase block, in a separate tube so that *no-RT control* reactions may be included in the QRT-PCR experiments. The RNase block serves as a safeguard against contaminating RNases.

AffinityScript RT is stringently quality-controlled to verify the absence of nuclease contaminants that adversely affect cDNA synthesis, particularly from small input RNAs. It has been tested in QRT-PCR experiments to ensure sensitive and reproducible performance over a broad range of RNA template amounts and over a variety of RNA targets that vary in size, abundance, and GC-content.

cDNA Primers

The cDNA priming strategy can affect cDNA yield, sensitivity, and detection of certain targets, such as GC-rich targets or sequences located at the 5' or 3' end of a transcript.[1] For this reason, individually packaged random nonamers and oligo(dT) primers are provided separately from the master mix, allowing you to use the best priming strategy for your specific target.[2]

QPCR Analysis using the Brilliant II QPCR Master Mix

The Brilliant II QPCR master mix includes all of the components necessary to carry out QPCR amplification of cDNA, including SureStart *Taq* DNA polymerase, an optimized buffer, dNTPs (GAUC), $MgCl_2$, and stabilizers. (Gene-specific primers and cDNA template are supplied by the user.) The Brilliant II master mix supports quantitative amplification and detection with multiplex capability and shows consistently high performance with various fluorescence detection systems, including molecular beacons and TaqMan® probes.

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 amplification reactions by decreasing background from non-specific amplification and increasing amplification of desired products. SureStart *Taq* is easily incorporated into PCR protocols already optimized with *Taq* DNA polymerase, with little modification of cycling parameters or reaction conditions.

Use of the Passive Reference Dye

A passive reference dye (an optional reaction component) is provided in a separate tube; providing this reagent separately allows you to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms.

QPCR Platforms

The Brilliant II QPCR master mix has been optimized for maximum performance on the Agilent Mx3000P and Mx3005P real-time PCR systems, as well as on the ABI 7900HT real-time PCR instrument. In addition, excellent results have been observed using most other QPCR platforms.

QPCR Probe Options

The Brilliant II QPCR master mix is designed for use with molecular beacons (Figure 1) or TaqMan (Figure 2) probes.

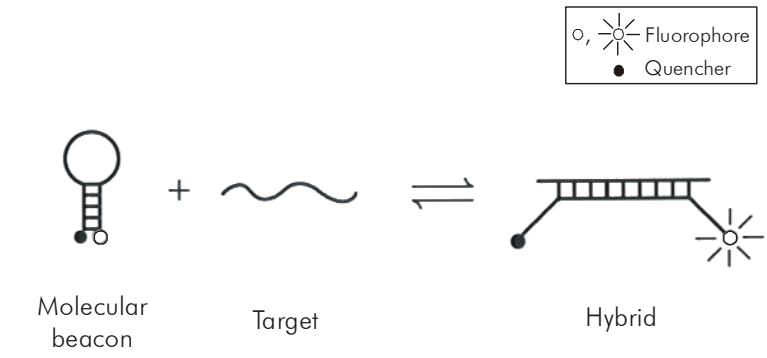

FIGURE 1 Mechanism of detection using molecular beacons probes. When the hairpin-structured molecular beacon is not bound to its target DNA, the fluorophore is quenched due to a close proximity to the quencher moiety. As target DNA is produced during PCR amplification, the molecular beacon binds to a complementary target, disrupting the hairpin structure, and allowing fluorescence.

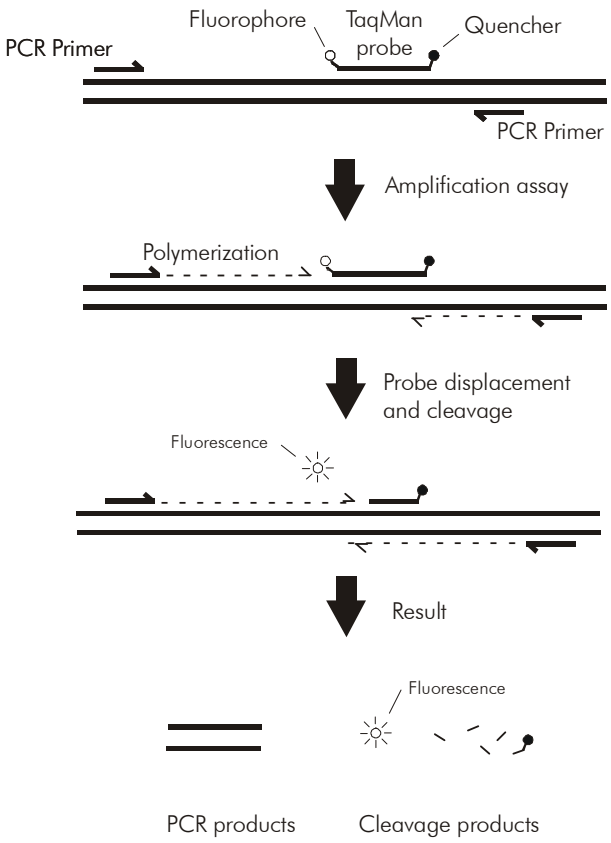

FIGURE 2 Mechanism of detection using TaqMan® probes. While the TaqMan probe is intact, no fluorescence is observed from the fluorophore, due to its close proximity to the quencher. During amplification, the 5′-nuclease activity of the DNA polymerase separates the fluorophore from the quencher, and fluorescence accumulates during the course of PCR.

PREPROTOCOL CONSIDERATIONS

RNA Isolation

High-quality intact RNA is essential for successful synthesis of full-length cDNA. Total RNA can be rapidly isolated and purified from cells using Agilent Absolutely RNA isolation kits, which are available for nano-, micro- and miniprep scale RNA purifications (Catalog #400753, #400805, and #400800, respectively). Total RNA may also be isolated from formalin-fixed, paraffin-embedded (FFPE) tissues using the Absolutely RNA FFPE kit (Catalog #400809).

Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although including this step may improve the yield of specific cDNA templates. The Absolutely mRNA purification kit (Catalog #400806) is recommended for this application.

For QRT-PCR experiments with mammalian cells, we offer the SideStep Lysis and Stabilization Buffer (Catalog #400900), which allows you to skip RNA isolation steps and analyze gene expression in cell lysates directly. Since the SideStep lysates also contain genomic DNA, it is critical to design PCR primers to span adjacent exons in order to selectively amplify cDNA and prevent amplification of the intron-containing genomic DNA.

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. The RNase block that is included in the cDNA synthesis reaction 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. All of the Agilent Absolutely RNA kits include RNase-free DNase which is used in a rapid on-column DNase treatment protocol. Additionally, PCR primers may be designed to span adjacent exons in order to prevent amplification of the intron-containing genomic DNA.

Using a Reference RNA for QRT-PCR Experiments

In order to reliably compare data across multiple experiments and instruments, it is essential to have a constant reference material to assess the performance of each QPCR run and to quantify gene expression levels. Agilent QPCR Reference Total RNA is a high-quality control for quantitative PCR gene-expression analysis. Including a standard curve with the Agilent QPCR Reference Total RNA in every experiment allows you to assess QPCR assay efficiency and precision, and to quantitate relative to an unchanging reference standard. In addition, the broad gene coverage allows you to use the reference material for nearly any human or mouse gene being investigated, thus eliminating the extra work required in generating new standards for each new gene target.

Agilent QPCR Reference Total RNAs are available for both the human system (Catalog #750500) and the mouse system (Catalog #750600). The QPCR Human Reference Total RNA is composed of total RNA from 10 human cell lines with quantities of RNA from the individual cell lines optimized to maximize representation of gene transcripts present in low, medium, and high abundance. The QPCR Mouse Reference Total RNA is derived from RNA pooled from 11 mouse cell lines. These reference RNAs are carefully screened for contaminating genomic DNA, the presence of which can complicate interpretation of QRT-PCR assay data.

cDNA Synthesis Reaction Considerations

Duration and Temperature of Incubation

For first-strand synthesis, a 15-minute incubation at 42°C is sufficient for most targets. Increasing the incubation time to 45 minutes at 42°C is optional and may increase cDNA yield for more challenging RNA targets, such as low-abundance targets or targets longer than 12 kb. For targets prone to secondary structure formation, raising the incubation temperature from 42°C to 55°C may improve cDNA yield.

Amount of AffinityScript RT

The cDNA synthesis reaction is fully optimized for high efficiency and dynamic range across a variety of targets and RNA input amounts. Do not try to address problems posed by low abundance or challenging targets by increasing the amount of AffinityScript RT/RNase block. (Use only 1 µl of AffinityScript RT/RNase block per 20-µl reaction.) See *Duration and Temperature of Incubation*, above, for recommendations on increasing cDNA synthesis incubation time or temperature to address yield problems for challenging targets. It is important to heat-inactivate the reverse transcriptase by incubating the reaction at 95°C for 5 minutes after cDNA synthesis.

Primer Selection

The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically with each target. Agilent QPCR Reference Total RNA (available separately) can be used for this step. (See *Using a Reference RNA for QRT-PCR Experiments* for more details.) For most targets, the best results are achieved using either oligo(dT) or random primers. For some challenging targets (long or secondary structure-rich targets), however, using a mixture of oligo(dT) and random primers may increase cDNA yield. When testing the use of mixed primers, adding a mixture of 170 ng oligo(dT) primer and 30 ng random primers to each 20-µl reaction is a good starting point.

Performing No-RT Control Reactions

Perform no-RT control reactions for each RNA sample by omitting AffinityScript reverse transcriptase from the reaction. The no-RT control is expected to generate no signal in subsequent QPCR if there is no amplification of genomic DNA. See *Preventing Genomic DNA Contamination* in *RNA Isolation*.

QPCR Reaction Considerations

Probe and PCR Primer Design

For best results, design the primers so that the PCR product is <150 bp in length.

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 beacons 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

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. TaqMan probe concentration can be optimized by varying the final concentration from 100 to 500 nM in increments of 100 nM. Molecular beacon concentration can be optimized by varying the final concentration from 200 to 500 nM in increments of 100 nM.

Optimal Concentrations for 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 TaqMan probes can be optimized by varying the concentration from 50 to 600 nM. The primer concentration for use with molecular beacons 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.

Using the Passive Reference Dye

The passive reference dye included in this kit 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 an Agilent Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a 1:500 dilution, resulting in a final concentration of 30 nM. If you are using the ABI 7900HT real-time PCR instrument, use the reference dye at a 1:50 dilution, resulting in 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 QPCR master mix contains $MgCl_2$ at a concentration of 5.5 mM (in the 1× solution), which is suitable for most targets.

Preventing Template Cross-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.

dUTP is used instead of dTTP in the Brilliant II QPCR master mix. When dUTP replaces dTTP in PCR amplification, treatment with UNG (Uracil-N-glycosylase, not provided in this kit) can prevent the subsequent reamplification of dU-containing PCR products. UNG acts on single- and double-stranded dU-containing DNA by hydrolysis of uracil-glycosidic bonds at dU-containing DNA sites. When this strategy is put to use, carry-over contamination will be eliminated while template DNA (DNA containing T) will be left intact.

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. Data should be collected at the annealing step of each cycle (3-step cycling protocol) or the annealing/extension step (2-step cycling protocol).

* 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.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[3] Multiplex PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.[4] The Brilliant II QPCR master mix has been successfully used to amplify two targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase or dNTPs.

In a typical multiplex PCR, one primer pair primes the amplification of the target of interest and another 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 for the more abundant target.[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 following guidelines are useful for multiplex PCR.

PCR Primer Considerations for Multiplex PCR

- Design primer pairs with similar annealing temperatures for all targets to be amplified.
- To avoid primer 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 20–200 nM is recommended. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.[5]

Probe Considerations for Multiplex PCR

Label each TaqMan probe or molecular beacon with a spectrally distinct fluorophore. The use of a dark quencher may enhance the quality of multiplex PCR results. Design molecular beacons for different targets to have different stem sequences.

PROTOCOL

Synthesis of First-Strand cDNA Using Reverse Transcriptase

Note *Before use, mix each component and spin in a microcentrifuge.*

It is prudent to include a No-RT Control *reaction for each RNA sample by omitting the AffinityScript RT/ RNase Block enzyme mixture. This control verifies that signal detected in the subsequent QPCR is not due to genomic DNA contamination.*

1. Prepare the first-strand cDNA synthesis reaction in a microcentrifuge tube by adding the following components *in order*:

   RNase-free H_2O to a total volume of 20 µl
   10.0 µl of cDNA synthesis master mix (2×)
   3.0 µl of oligo(dT) primer OR random primers (0.1 µg/µl)
   1.0 µl of AffinityScript RT/ RNase Block enzyme mixture
   X µl of RNA (0.3 pg–3 µg total RNA)

Note *The optimum primer type [oligo(dT) or random primer] varies for different targets and should be determined empirically. See* Primer Selection *in* Preprotocol Considerations *for more information.*

2. Incubate the reaction at 25°C for 5 minutes to allow primer annealing.

3. Incubate the reaction at 42°C for 15 minutes to allow cDNA synthesis.

   Notes *This protocol has been extensively tested and is ideal for most targets up to 12 kb. Increasing the incubation time to 45 minutes at 42°C or raising the incubation temperature from 42°C to 55°C may increase cDNA yield for longer or secondary structure-rich targets, respectively.*

   When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C for first-strand synthesis.

4. Incubate the reactions at 95ºC for 5 minutes to terminate the cDNA synthesis reaction.

5. Place the completed first-strand cDNA synthesis reactions on ice for immediate use in QPCR. For long-term storage, place the reactions at –20°C.

QPCR Amplification of cDNA

Notes *Once the tube containing the Brilliant II QPCR 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. 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.

Consider performing an endogenous control reaction to distinguish true negative results from PCR inhibition or failure. For information on the use and production of endogenous controls for QPCR, see Reference 6.

Setting Up the QPCR 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 *Using the Passive Reference Dye* under *Preprotocol Considerations*. When used according to the protocol below, this will result in a final reference dye concentration of 30 nM for the 1:500 dilution and 300 nM for the 1:50 dilution. **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 experimental reaction by adding the following components *in order*:

   Experimental Reaction

   Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including cDNA, added in step 4)
   12.5 µl of 2× 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)

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

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

4. Add 2 µl of the cDNA synthesis reaction.

5. Gently mix the reaction without creating bubbles (do not vortex).

   Note *Bubbles interfere with fluorescence detection.*

6. Centrifuge the reaction briefly.

PCR Cycling Programs

7. Place the reaction in the instrument and run the appropriate PCR program below. Generally, TaqMan reactions utilize the 2-step cycling program, and molecular beacons reactions utilize the 3-step cycling program. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

Two-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	15–30 seconds	95°C
	1.0 minute[b]	55–60°C[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.

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

Three-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1.0 minute[b]	55–60°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 step of each cycle.

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

TROUBLESHOOTING

Observation	Suggestion
No or low yield of first-strand cDNA	Verify the integrity of the input RNA by denaturing agarose gel electrophoresis to ensure it is not degraded.
	Optimize the reaction using Agilent QPCR Reference Total RNAs, which can then be used as a calibrator for subsequent experiments.
	Prepare a new RNA sample. Use Agilent RNA isolation kits to isolate intact total RNA or mRNA.
	Isolate the RNA in the presence of a ribonuclease inhibitor, and ensure that all RT-PCR reagents and labware are free of RNases.
	Inhibitors of reverse transcription (SDS, EDTA, guanidinium chloride, formamide, Na_2PO_4, or spermidine) may be present in the RNA sample. Reduce the volume of the input RNA or remove RT inhibitors with an additional 70% (v/v) ethanol wash following ethanol precipitation.
	Increase the length of the 42°C cDNA synthesis reaction to 45 minutes for challenging RNA targets.
	Increase the incubation temperature of the cDNA synthesis reaction from 42°C to 55°C for secondary structure-rich targets.
	Increase the concentration of the template RNA.
	Try switching the cDNA primer composition [oligo(dT) vs. random primers]. For challenging targets, a mixture of the two primer types may also be tested.
	When using oligo(dT) to prime cDNA synthesis at an incubation temperature of 55°C, incubate the reactions at 42°C for 5 minutes before transferring to 55°C for first-strand synthesis.
No or low yield of amplification product in QPCR	See the discussion under *No or low yield of the first-strand cDNA* for suggestions related to insufficient first strand synthesis.
	Add more cDNA synthesis product to the PCR. Up to 10% of the cDNA synthesis reaction may be added to each 25-µl QPCR reaction.
	Optimize the QPCR primer concentration, annealing temperature, and/or extension time, varying each individually and in increments. Agilent QPCR Reference Total RNAs may be used for this step to conserve experimental samples.
	Increase the number of thermal cycles.
	Re-examine the QPCR primer design. For best results, design primers so that amplicons are <150 bp in length. Make sure primers are not self-complementary or complementary to each other. Verify that the primers are designed to be complementary to the appropriate strands. Try using longer primers.
	Re-examine the probe design. Consult a primer/probe design software tool, such as the Primer Express® oligo design software from Applied Biosystems. Design TaqMan probes that are compatible with 5.5 mM $MgCl_2$ and with melting temperatures 7–10°C higher than the annealing temperature of the primers.
	Verify the function of the probe's fluorophore. For molecular beacons, fluorophore function is confirmed by an increase in fluorescence in the denaturation step of thermal cycling or at high temperatures in a melting curve analysis. For TaqMan probes, 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. If increased fluorescence is not observed, resynthesize the probe or molecular beacon.

Table continues on following page

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No or low yield of amplification product in multiplex QPCR	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.
	For multiplex PCR of more than two targets, the master mix may need to be supplemented with additional polymerase and dNTPs (not provided in this kit).
Size of the amplification product is greater than expected	The RNA preparation may be contaminated with genomic DNA. Test for the presence of contaminating DNA by performing RT-PCR in the absence of AffinityScript RT. If DNA contamination is confirmed, treat the RNA preparation with RNase-free DNase I. Alternatively, redesign the PCR primers to anneal to sequences in the exon–exon boundary of the target gene.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 7 to minimize contamination.
	Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See *Preventing Template Cross Contamination* in *Preprotocol Considerations*.
Ct reported for the no-target 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.
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 II QRT-PCR, AFFINITYSCRIPT TWO-STEP MASTER MIX

Catalog #600827

QUICK-REFERENCE PROTOCOL

cDNA Synthesis Reaction

1. Add the following components to a microcentrifuge tube *in order:*
   RNase-free H_2O to a total volume of 20 µl
   10.0 µl of cDNA synthesis master mix (2×)
   3.0 µl of oligo(dT) primer OR random primers (0.1 µg/µl)
   1.0 µl of AffinityScript RT/ RNase Block enzyme mixture
   X µl of RNA (0.3 pg–3 µg total RNA)

2. Incubate the reaction at 25°C for 5 minutes to allow primer annealing.

3. Incubate the reaction at 42°C for 15 minutes to allow cDNA synthesis.

   Note *For challenging targets, increasing the incubation time to 45 minutes at 42°C or raising the incubation temperature from 42°C to 55°C may increase cDNA yield.*

4. Incubate the reaction at 95°C for 5 minutes to terminate the cDNA synthesis reaction.

5. Place the reaction on ice for immediate use in QPCR or at –20°C for long-term storage.

QPCR Reactions

1. If the passive reference dye will be included in the reactions (optional), dilute 1:500 (Mx3000P, Mx3005P, or Mx4000 instruments) or 1:50 (ABI 7900HT real-time PCR 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 Brilliant II QPCR master mix and store on ice. Following initial thawing of the master mix, store the unused portion at 4°C.

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

Reagent Mixture

Nuclease-free PCR-grade H_2O to bring the final volume to 25 µl (including cDNA)
- 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 (optional)

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

5. Add 2 µl of cDNA 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

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	15–30 seconds[b]	95°C
	1.0 minute[c]	55–60°C[d]

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

[b] For the Mx4000 instrument, use 30 seconds; for the Mx3000P, Mx3005P, and ABI 7900HT real-time PCR instruments, use 15 seconds.

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

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

Three-step Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	30 seconds	95°C
	1.0 minute[b]	55–60°C[c]
	30 seconds	72°C

[a] An 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 step of each cycle.

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