Brilliant II QPCR Master Mix with ROX

Instruction Manual

Catalog #600805 (Brilliant II QPCR Master Mix with High ROX)

#600806 (Brilliant II QPCR Master Mix with Low ROX)

#600816 (Brilliant II QPCR Master Mix with High ROX, 10-pack kit)

#600817 (Brilliant II QPCR Master Mix with Low ROX, 10-pack kit)

Revision D

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600805-12

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Brilliant II QPCR Master Mix with ROX

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MATERIALS PROVIDED

Materials provided	Catalog #600805[a]	Catalog #600806[a]	Catalog #600816	Catalog #600817
2× Brilliant II QPCR High ROX Master Mix	2 × 2.5 ml	—	20 × 2.5 ml	—
2× Brilliant II QPCR Low ROX Master Mix	—	2 × 2.5 ml	—	20 × 2.5 ml

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

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 Brilliant II QPCR master mix with ROX reference dye is light sensitive; solutions containing the master mix should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

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 D

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 QPCR Master Mix with ROX is a single-tube reagent ideal for most high-throughput QPCR applications. Brilliant II 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 master mix includes the components necessary to carry out QPCR amplifications*, and has been successfully used to amplify and detect a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA.

The Brilliant II QPCR master mix with ROX is available in two formulations: a high ROX master mix and a low ROX master mix. The final concentration of ROX reference dye in QPCR reactions prepared with the high ROX master is 500 nM. Reactions prepared with the low ROX master mix contain 30 nM of ROX. The two formulations are recommended for different real-time PCR platforms. Refer to the table below for catalog information and a list of compatible instruments for each product.

Catalog #	Product Name	Final ROX concentration	Compatible instruments
600805 (10-pack, 600816)	Brilliant II QPCR Master Mix with High ROX	500 nM	ABI PRISM® 7000 and 7700; ABI 7300, 7900HT and 7900HT Fast systems
600806 (10-pack, 600817)	Brilliant II QPCR Master Mix with Low ROX	30 nM	Stratagene Mx3000P, Mx3005P and Mx4000 systems; ABI 7500 system

ROX dye is included in the master mix to compensate for non-PCR related variations in fluorescence. Fluorescence from ROX dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, ROX detection compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of ROX dye are 584 nm and 612 nm, respectively.

In addition to ROX, the QPCR master mix includes SureStart *Taq* DNA polymerase, nucleotides (GUAC), and an optimized QPCR buffer. SureStart *Taq* DNA polymerase, a modified version of *Taq2000* DNA polymerase with hot start capability, improves PCR amplification reactions by decreasing background from nonspecific amplification 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 modification of cycling parameters or reaction conditions.

* 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 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. 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 (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. 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.

Endpoint vs. Real-Time Measurements

The probes can be used in a variety of PCR applications, including infectious agent detection, genotyping, detection of single nucleotide polymorphism (SNP) variants, allelic discrimination, and quantitative gene expression analysis. 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.

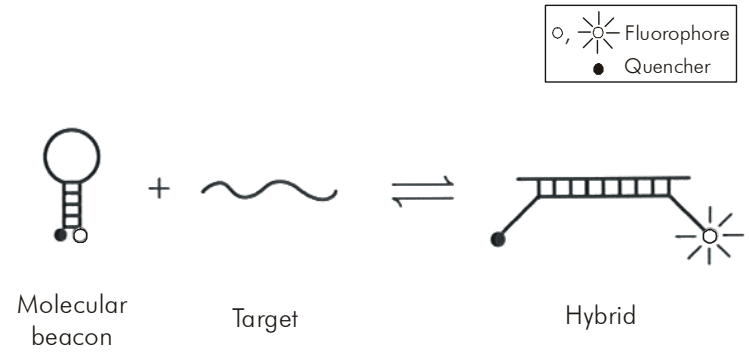

FIGURE 1 The molecular beacon binds to a complementary target and fluoresces.

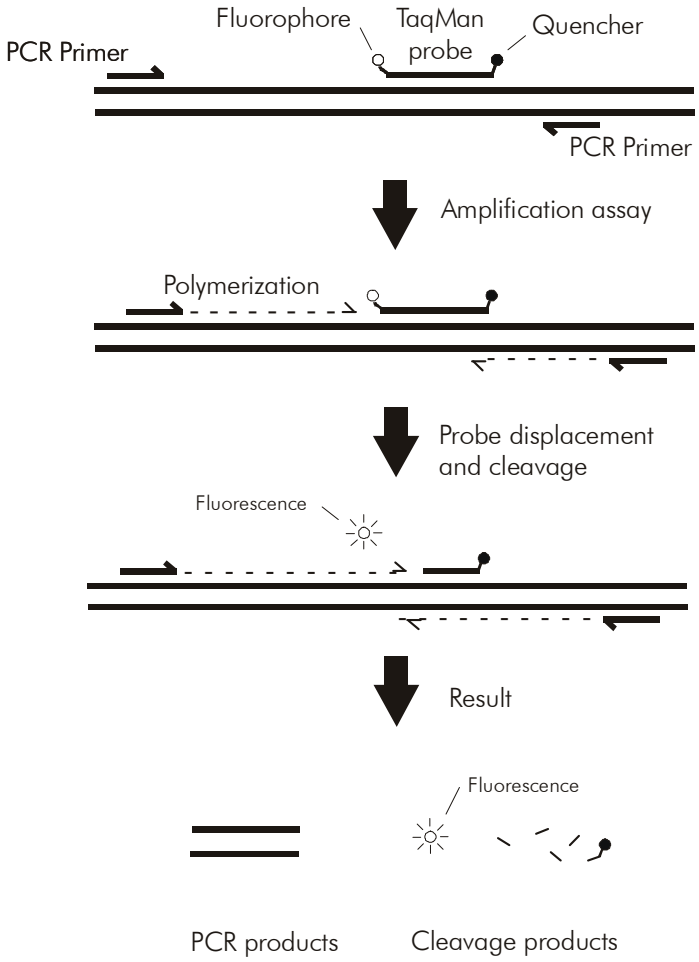

FIGURE 2 TaqMan probe fluoresces when the 5′-nuclease activity of the DNA polymerase separates the fluorophore from quencher.

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 PCR experiments to quantitate initial copy number. Quantitative assessments based on endpoint fluorescence values (a single reading taken at the end of the PCR reaction) are inherently inaccurate because endpoint values can be greatly influenced by limiting reagents and small differences in reaction components or cycling parameters. Studies have shown that initial copy number can be quantitated during real-time PCR analysis based on 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. Figure 3 shows an Mx3005P instrument amplification plot (top panel) and standard curve (bottom panel). In this experiment, the β-actin gene was amplified and detected using a TaqMan probe and the Brilliant II QPCR low ROX master mix.

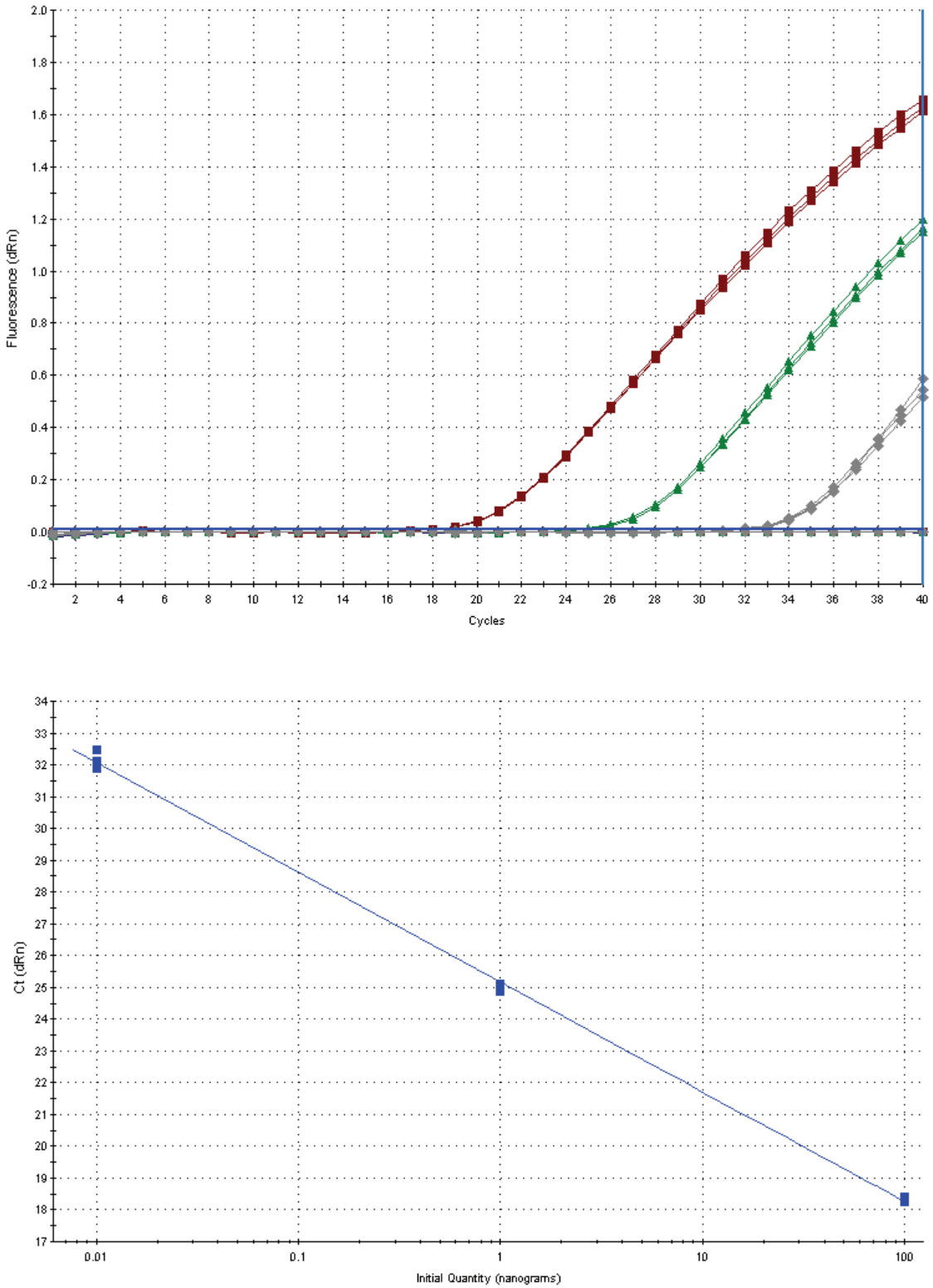

Figure 3 *Top panel*: Mx3005P quantitative PCR instrument amplification plot using a TaqMan® probe. A serial dilution of genomic DNA template was added to each reaction and reactions were performed in triplicate. The amount of DNA template added per reaction (in ng) is indicated to the left of each set of curves. The fluorescence value used to determine Ct (the threshold line) is shown as a solid line. *Bottom panel*: Standard curve generated from amplification plot. An amplification efficiency of 94.8% and an RSq value of 0.999 were obtained.

PREPROTOCOL CONSIDERATIONS

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 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 50 to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

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).

Fluorescence Detection

Fluorescence may be detected either in real-time or at the endpoint of cycling using a real-time spectrofluorometric thermal cycler. 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 reader, it is recommended that readings be taken both before and after PCR for comparison.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[8] Multiplex PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.[9] 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.[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 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 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.[10]

Probe Considerations for Multiplex PCR

A) Molecular Beacons

- Label each molecular beacon with a spectrally distinct fluorophore.[11] 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.

B) TaqMan® Probes

Label each TaqMan probe with a spectrally distinct fluorophore. The use of a dark quencher may enhance the quality of multiplex PCR results.

PROTOCOL

Preparing the Reactions

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. The master mix with ROX reference dye is light sensitive; solutions containing the master mix should be kept away from light whenever possible. 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 12.

1. Prepare the experimental reactions by adding the following components *in order*. We recommend preparing 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 DNA)
12.5 µl of 2× QPCR master mix
x µl of experimental probe (optimized concentration)
x µl of upstream primer (optimized concentration)
x µl of downstream primer (optimized concentration)

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

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

3. Add *x* µl of experimental gDNA, cDNA, or plasmid DNA to each reaction.

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

Note *Bubbles interfere with fluorescence detection.*

5. Centrifuge the reactions briefly.

PCR Cycling Programs

6. Place the reactions in the instrument and run the appropriate PCR program below. Generally, TaqMan reactions utilize the 2-step program, and molecular beacons reactions utilize the 3-step 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]	60°C

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

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

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 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: 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 calculated 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.
	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 enough specific product by gel electrophoresis.
	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).
There is increased fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 13 to minimize contamination.
	Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See *Preventing Template Cross Contamination* in *General Notes*.
Ct reported for the no-target control (NTC) sample 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.

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.
	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 enough specific product by gel electrophoresis.
	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).
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.
	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 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.

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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 QPCR MASTER MIX

Catalog #600805, #600806, #600816, #600817

QUICK-REFERENCE PROTOCOL

1. Thaw the 2× Brilliant II QPCR 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.*

2. Prepare the experimental 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 DNA)
   - 12.5 µl of 2× QPCR master mix
   - x µl of experimental probe (optimized concentration)
   - x µl of upstream primer (optimized concentration)
   - x µl of downstream primer (optimized concentration)

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

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

4. Add x µl of experimental gDNA, cDNA, or plasmid DNA to each reaction.

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

6. Centrifuge the reactions briefly.

7. 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]	60°C

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

[b] For the Mx4000 instrument, use 30 seconds; for the Mx3000P, Mx3005P, and ABI PRISM 7700 instruments, use 15 seconds.

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

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 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.