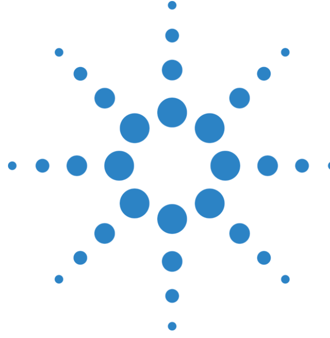

Brilliant II Fast QPCR Master Mix

Instruction Manual

Catalog #600845 (single kit)
#600846 (10-pack kit)

Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

600845-12

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Brilliant II Fast QPCR Master Mix

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Brilliant II Fast QPCR Master Mix

MATERIALS PROVIDED

Catalog #600845 (single kit), #600846 (10-pack kit)

Materials provided	Quantity [a,b]
2× Brilliant II Fast QPCR Master Mix	2 × 2.5 ml
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: Upon receipt, store all components at –20°C. After thawing, the 2× master mix may be stored at 4°C for up to one month or returned to –20°C for long term storage.

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

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

NOTICE TO PURCHASER

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.

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INTRODUCTION

The Brilliant II Fast QPCR Master Mix is a single-tube reagent designed for performing quantitative PCR amplifications with an accelerated cycling protocol that completes 40 cycles of PCR in approximately 48 minutes*, yielding results in half the time of standard QPCR protocols. The master mix formulation and fast cycling protocol were developed together to create a system for completing QPCR reactions in less time without compromising target detection sensitivity, specificity, or reproducibility. The Brilliant II Fast QPCR master mix has been successfully used with fluorescent TaqMan® probes to amplify and detect a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA.

The 2× master mix contains *Taq* DNA polymerase, dNTPs, Mg^{2+}, and a buffer specially formulated for fast cycling. The DNA polymerase features a rapid hot start capability that reduces nonspecific product formation while keeping the run time of the PCR protocol to a minimum, making it ideal for the Brilliant II Fast QPCR kits.

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

The master mix has been optimized for maximum performance on the Stratagene Mx3000P and Mx3005P real-time PCR systems and Stratagene Mx4000 multiplex quantitative PCR system, as well as on the ABI 7900HT real-time PCR instrument. In addition, excellent results have been observed using other QPCR platforms.

TaqMan® Probes (Hydrolysis Probes)

TaqMan probes are linear.[1, 2] 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 1). 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. 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.

* Based on performance using the Mx3005P QPCR system.

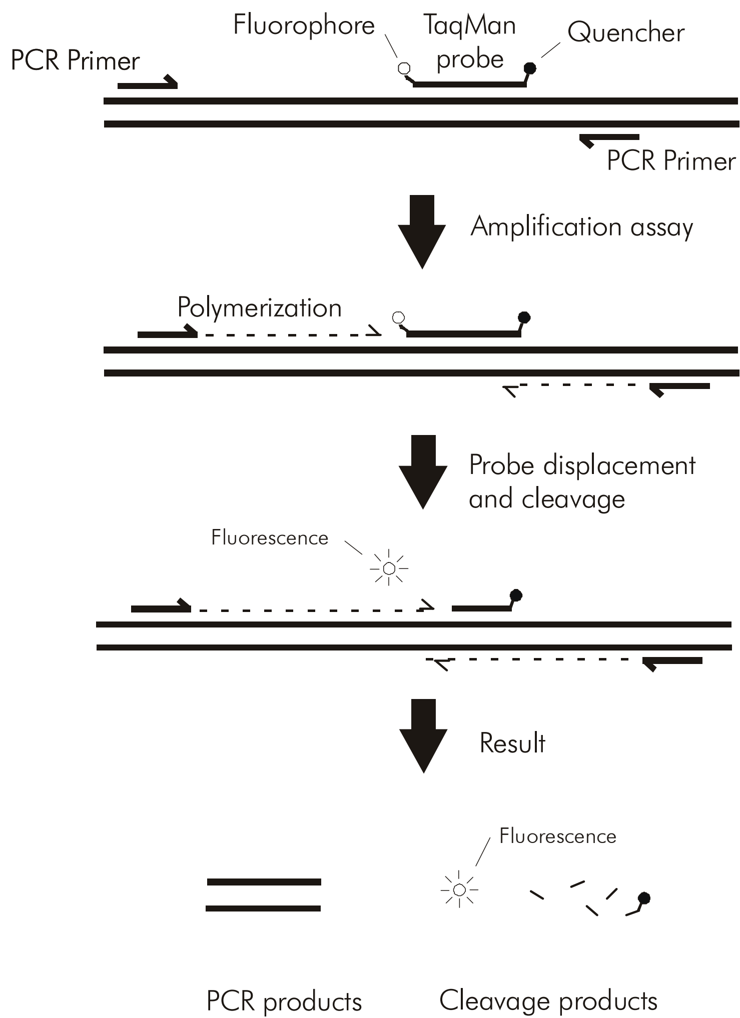

FIGURE 1 TaqMan probe fluoresces when the 5´-nuclease activity of the DNA polymerase separates the fluorophore from quencher.

Endpoint vs. Real-Time Measurements

The fluorescence of the TaqMan probe can be monitored either when cycling is complete (endpoint analysis) or as the reaction is occurring (real-time analysis). Real-time experiments are typically performed on an instrument capable of detecting fluorescence from samples during each cycle of a PCR protocol. Acquisition of real-time data should be performed as recommended by the instrument's manufacturer. Data should be collected at the annealing/extension step of each cycle.

For endpoint analysis, PCR reactions can be run on any thermal cycler and then analyzed with a fluorescence plate reader designed to accommodate PCR tubes and optimized for the detection of PCR reactions that include fluorescent probes. If using a fluorescence plate reader, it is recommended that readings be taken both before and after PCR for comparison.

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. Studies have shown that initial copy number can be quantitated during real-time PCR analysis based on threshold cycle (Ct).[3] 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.[3] The more template that is initially present, the fewer the number of cycles it takes to reach the point where the fluorescence signal is detectable above background. Quantitative information based on threshold cycle is more accurate than information based on endpoint determinations because threshold cycle is based on measurements taken during the exponential phase of PCR amplification when PCR efficiency is not yet influenced by limiting reagents, small differences in reaction components, or accumulation of PCR inhibitors. Figure 2 shows an Mx3005P instrument amplification plot with Ct determination (top panel) and standard curve (bottom panel). In this experiment, the “housekeeping” gene GUS was amplified and detected using a TaqMan probe and the Brilliant II Fast QPCR master mix.

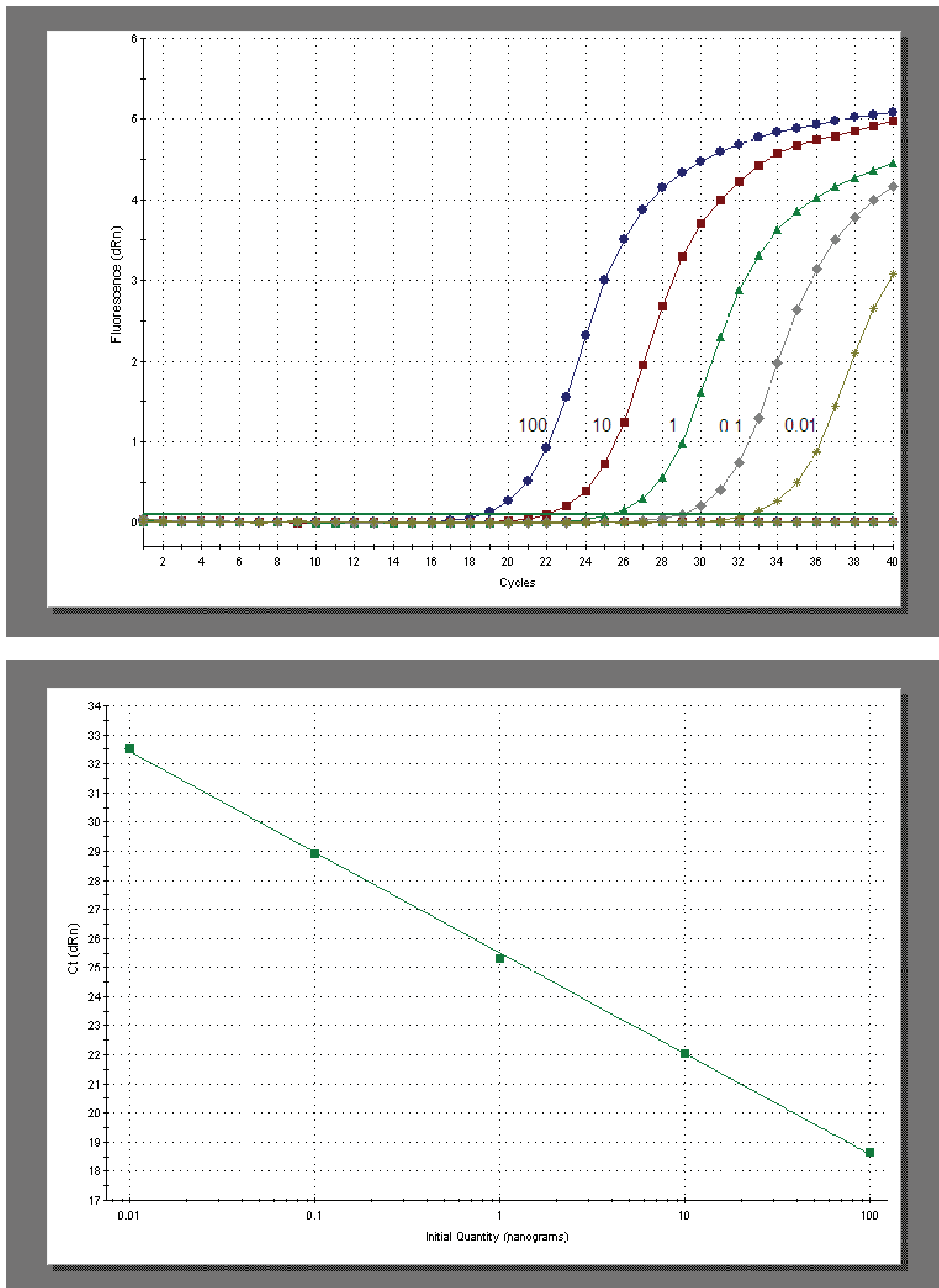

Figure 2 *Top panel*: Mx3005P QPCR instrument amplification plot using a TaqMan® probe. A serial dilution of cDNA template was added to each reaction and reactions were performed in triplicate (average Ct is displayed). The amount of cDNA template added per reaction (in ng) is indicated to the left of each amplification curve. 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 94.5% and an RSq value of 1.000 were obtained.

PREPROTOCOL CONSIDERATIONS

Magnesium Chloride

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

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 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 TaqMan probe should be determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The TaqMan probe concentration can be optimized by varying the final concentration from 200 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 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.

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 Mx3005P, Mx3000P or Mx4000 system, with other instruments (including the ABI 7900HT) the use of the reference dye may be required for optimal results.

Reference Dye Dilution Recommendations

Prepare **fresh*** dilutions of the reference dye prior to setting up the reactions, and **keep all tubes containing the reference dye protected from light as much as possible**. Make initial dilutions of the reference dye using nuclease-free PCR-grade H_2O. If using a Stratagene Mx3000P, Mx3005P, or Mx4000 instrument, use the reference dye at a final concentration of 30 nM. If using the ABI 7900HT real-time PCR instrument, use the reference dye at a final concentration of 300 nM. For other instruments, use the following guidelines for passive reference dye optimization. For instruments that allow excitation at ~584 nm (including most tungsten/halogen lamp-based instruments and instruments equipped with a ~584 nm LED), begin optimization using the reference dye at a final concentration of 30 nM. For instruments that do not allow excitation near 584 nm, (including most laser-based instruments) begin optimization using the reference dye at a final concentration of 300 nM.

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 Fast 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.

* The diluted reference dye, if stored in a light-protected tube at 4°C, can be used within the same day for setting up additional assays.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[4] Multiplex PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.[5] The Brilliant II Fast 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.[6] 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.

Probe Considerations for Multiplex PCR

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

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 50–200 nM is recommended. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.[6]

PROTOCOL

Preparing the Reactions

Notes *Once the tube containing the Brilliant II Fast 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 for up to one month or –20°C for long term storage.*

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 3.

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

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

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

Reagent Mixture

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental DNA)
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 reagents without creating bubbles (do not vortex), then distribute the mixture to the experimental reaction tubes.

4. Add *x* µl of experimental DNA to each reaction. The table below lists a suggested quantity range for different DNA templates.

DNA	Quantity per reaction
Genomic DNA	5 pg–100 ng
cDNA	1 pg–100 ng*

* Refers to RNA input amount during cDNA synthesis

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

Note *Bubbles interfere with fluorescence detection.*

6. Centrifuge the reactions briefly.

PCR Cycling Programs

7. Place the reactions in the instrument and run the PCR program below.

Brilliant II Fast Cycling Protocol[a]

Cycles	Duration of cycle	Temperature
1	2 minutes[b]	95°C
40	5 seconds	95°C
	20 seconds[c]	60°C

[a] This protocol has been optimized for use on the Stratagene Mx3000P, Mx3005P and Mx4000 QPCR systems, as well as the ABI 7900HT real-time PCR instrument.
[b] Initial 2-minute incubation is required to activate the DNA polymerase.
[c] Set the temperature cycler to detect and report fluorescence during the annealing/extension step of each cycle.

TROUBLESHOOTING

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 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 DNA polymerase was not activated. Ensure that the 2-minute initial incubation at 95°C was performed as part of the cycling parameters.
	The DNA polymerase was activated for more than 2 minutes. Ensure that the initial 95°C incubation was not longer than 2 minutes.
	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 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 *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.

References

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Endnotes

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

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

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BRILLIANT II FAST QPCR MASTER MIX

Catalog #600845, #600846

QUICK-REFERENCE PROTOCOL

1. If the passive reference dye will be included in the reaction (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 Fast QPCR master mix and store on ice. Following initial thawing of the master mix, the unused portion may be stored at 4°C for up to one month or returned to –20°C for long term storage.

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

3. Prepare the experimental reaction 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)
   0.375 µl of diluted reference dye (optional)

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

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

5. Add x µl of genomic DNA, cDNA, or plasmid DNA to the reaction.

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

7. Centrifuge the reaction briefly.

8. Place the reaction in the instrument and run the PCR program below.

Cycles	Duration of cycle	Temperature
1	2 minutes[a]	95°C
40	5 seconds	95°C
	20 seconds[b]	60°C

[a] An initial 2-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.