Brilliant Multiplex QPCR Master Mix

Instruction Manual

Catalog #600553

Revision D.0

For Research Use Only. Not for use in diagnostic procedures.

600553-12

Agilent Technologies

LIMITED PRODUCT WARRANTY

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

ORDERING INFORMATION AND TECHNICAL SERVICES

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit *www.agilent.com/genomics/contactus*

Brilliant Multiplex QPCR Master Mix

CONTENTS

Brilliant Multiplex QPCR Master Mix

MATERIALS PROVIDED

Materials provided[a]	Concentration	Quantity
2× Multiplex QPCR master mix	2×	2.5 ml
Reference dye[b]	1 mM	100 µl

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

[b] The reference dye is light sensitive and should be kept away from light whenever possible.

STORAGE CONDITIONS

All Components: Upon receipt, store all components at –20°C. Store the 2× master mix at 4°C after thawing. Once thawed, full activity is guaranteed for 6 months.

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

NOTICES TO PURCHASER

NOTICE TO PURCHASER: LIMITED LICENSE
Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 6,258,569, 6,171,785, 6,127,155, 6,030,787, 5,994,056, 5,876,930, 5,804,375, 5,789,224, 5,773,258 (claims 1 and 6 only), 5,723,591, 5,677,152 (claims 1 to 23 only), 5,618,711, 5,538,848, and claims outside the US corresponding to expired US Patent No. 5,079,352. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Revision D.0

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.

Real-time quantitative PCR can provide both absolute and relative quantification during the exponential phase of amplification. To achieve accurate quantification, the use of a normalizing gene is required. Multiplex QPCR allows one or more targets of interest to be amplified with a normalizing gene in the same reaction vessel by using probes with spectrally different fluorophores for the detection of each amplicon. Unlike conventional QPCR reagents which can amplify up to two targets, Agilent's Brilliant Multiplex QPCR Master Mix is specifically optimized to amplify up to four targets in one reaction without sacrificing the accuracy of gene transcript quantification.

The Brilliant multiplex QPCR master mix is provided in a single-tube reagent format, making it ideal for most high-throughput QPCR applications. The Brilliant multiplex master mix supports quantitative amplification and detection with multiplex capability and shows consistent high performance with various fluorescent detection systems, including molecular beacons and TaqMan® probes. The Brilliant multiplex master mix includes the components necessary to carry out PCR amplifications,* and has been successfully used to amplify and detect a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA. Including the passive reference dye in a separate tube makes the Brilliant multiplex master mix adaptable for many real-time QPCR platforms.

* Primers and template are not included.

The Brilliant multiplex master mix provides amplification of multiple targets per reaction. The sensitivity is equivalent to that seen in singleplex QPCR reactions, maximizing use of each precious sample without sacrificing sensitivity (see Figure 1). In addition, the Brilliant multiplex master mix provides sufficient resources to allow amplification of a low abundance target and a high abundance target in the same tube.

The Brilliant multiplex QPCR master mix 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 from non-specific amplification. 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.

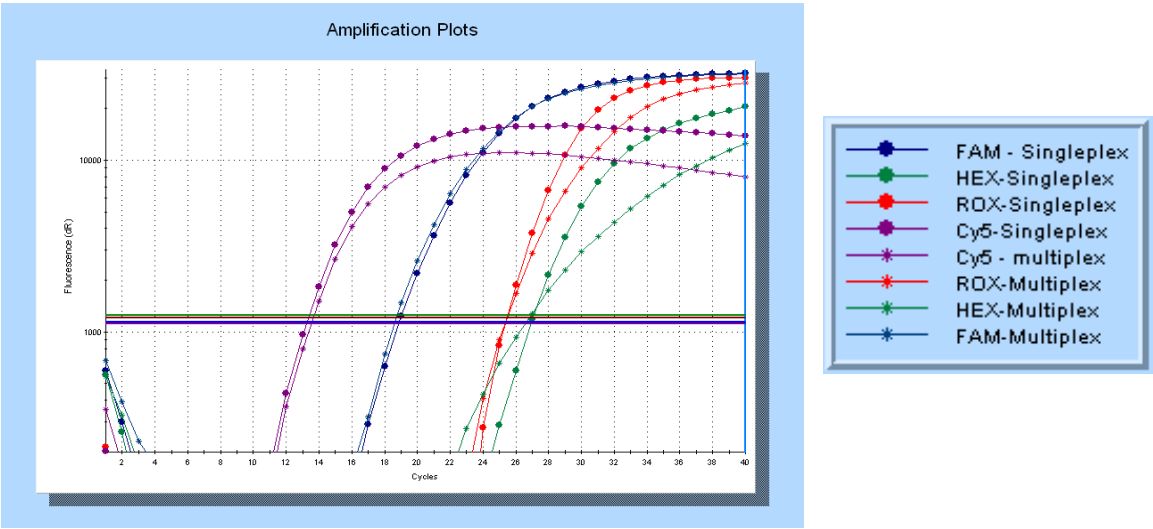

FIGURE 1 Four targets – eNOS (FAM), HFE (HEX), CFTR (TR), and Cyclophilin (Cy5) – were run in singleplex and multiplex using the Brilliant Multiplex QPCR master mix on the Mx3000P real-time PCR system. Ct values for each target were virtually identical in the two reactions, indicating full sensitivity and performance in multiplex.

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 2). 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

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 3). 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.

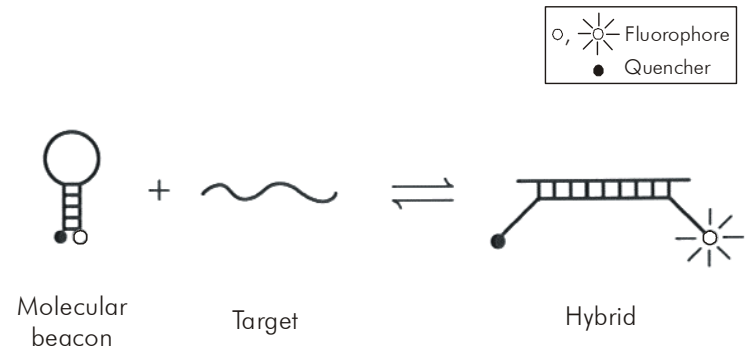

FIGURE 2 The molecular beacon binds to a complementary target and fluoresces.

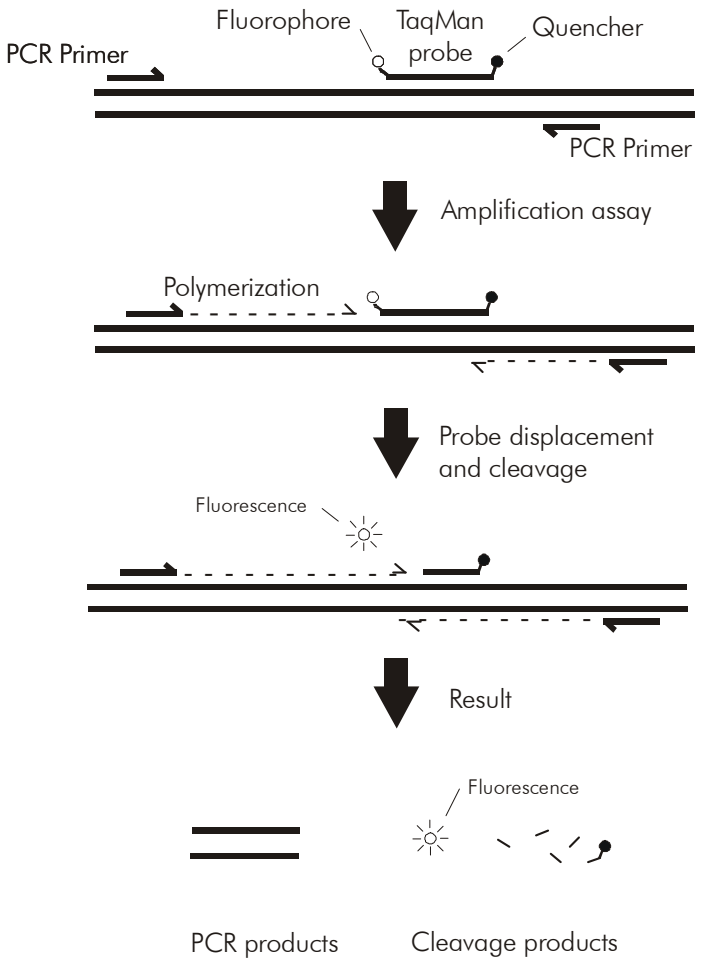

FIGURE 3 TaqMan probe fluoresces when the 5′-nuclease activity of the DNA polymerase separates the fluorophore from quencher.

Endpoint vs. Real-Time Measurements

Both molecular beacons and linear fluorescent probes can be used in a variety of PCR applications, including infectious agent detection, genotyping, 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.

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 (see Figure 1), 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.

PREPROTOCOL CONSIDERATIONS

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 multiplex QPCR master mix has been successfully used to amplify four targets in a multiplex reaction without reoptimizing the concentrations of DNA polymerase or dNTPs. 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 optimal concentration of the upstream and downstream PCR primers 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 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.
- To minimize competition, the limiting primer concentrations need to be determined for the more abundant target.[10] 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 multiple targets to be amplified is known, determine the limiting primer concentrations for the most abundant target. If the relative abundance of the multiple targets is unknown, determine the limiting primer concentrations for each target. 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. Running duplicates or triplicates of each combination of primer concentrations within the matrix is also recommended.[10]

Multiplex PCR continued

Probe Considerations for Multiplex PCR

- 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.
- Label each probe 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.
- 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. The molecular beacon or TaqMan probe concentration can be optimized by varying the final concentration from 100 to 300 nM in increments of 100 nM.
- 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).

Magnesium Chloride Concentration

Magnesium chloride concentration affects the specificity of the PCR primers and probe hybridization. The Brilliant multiplex 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.

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. When using the passive reference dye, the choice of experimental fluorophores must be considered. If any of the fluorophores used has excitation and emission wavelengths close to 584 and 612, respectively, use of the passive reference dye is not recommended. 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 or Mx3005P real-time PCR systems or a Mx4000 multiplex quantitative PCR system, use the reference dye at a final concentration of 30 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.

Fluorescence Detection

Fluorescence may be detected either in real-time or at the endpoint of cycling using a real-time spectrofluorometric thermal cycler.

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 (three-step cycling protocol) or the annealing/extension step (two-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.

Notes *Once the tube containing the Brilliant multiplex 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.

1. If the reference dye will be included in the reactions (optional), dilute the dye solution provided 1:500 using nuclease-free PCR-grade H_2O. Keep all solutions containing the reference dye protected from light.

   Notes *Inclusion of passive reference dye is advisable only if the fluorophores used in multiplex reaction do not have excitation and emission wavelengths similar to 584 and 612 respectively.*

   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 multiplex QPCR master mix and store the tube on ice. Following initial thawing of the master mix, store the unused portion at 4°C.

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

3. Prepare each 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 experimental DNA)
   12.5 µl of 2× master mix
   x µl of each experimental probe (optimized concentration)
   x µl of each upstream primer (optimized concentration)
   x µl of each downstream primer (optimized concentration)
   0.375 µl of the 1:500 dilution of reference dye (**optional**) (final concentration=30 nM)

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

4. Gently mix the reactions without creating bubbles (do not vortex). Add *x* µl of experimental gDNA, cDNA, or plasmid DNA to each experimental reaction.

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

   Note *Bubbles interfere with fluorescence detection.*

6. Place the reactions in the instrument and run the appropriate PCR program below. Generally, TaqMan and other hydrolysis probe reactions utilize the two-step program, and molecular beacons reactions utilize the three-step program. These amplification protocols are recommended initially, but optimization may be necessary for some primer/template systems.

   Note *Optimal cycling programs will differ from those given below when using lamp-based instruments other than the Mx4000, Mx3000P, and Mx3005P instruments.*

Two-Step PCR Cycling Protocol

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

Three-Step PCR Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	15 seconds	95°C
	1.0 minute[b]	55–60°C[c]
	30 seconds	72°C

[a] The 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: 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 loop sequence.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
Efficiency of the singleplex reactions are not the same as the multiplex reactions	Optimize the primer and probe concentrations to give similar efficiencies when in singleplex or multiplex.
There is an increase in fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 12 to minimize contamination.
	Probe degradation may be occurring.
Ct reported for the no-template control (NTC) wells is less than the total number of cycles but the amplification plot is relatively flat (i.e., it does not appear to be specific amplification)	Review the amplification plot and, if appropriate, adjust the threshold accordingly.

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 sequence.
	The reaction is not optimized and no or insufficient product is formed. Verify formation of enough specific product by gel electrophoresis.
Efficiency of the singleplex reactions are not the same as the multiplex reactions	Optimize the primer and probe concentrations to give similar efficiencies when in singleplex or multiplex.
There is increased fluorescence in control reactions without template	The reaction has been contaminated. Follow the procedures outlined in reference 12 to minimize contamination.
	Probe degradation may be occurring.
Ct reported for the no-template control (NTC) wells is less than the total number of cycles but the amplification plot is relatively flat (i.e., it does not appear to be specific amplification)	Review the amplification plot and, if appropriate, adjust the threshold accordingly.

REFERENCES

1. Kostrikis, L. G., Tyagi, S., Mhlanga, M. M., Ho, D. D. and Kramer, F. R. (1998) *Science* 279(5354):1228-9.
2. Padmabandu, G., Grismer, L. and Mueller, R. (2000) *Strategies* 13(3):88-92.
3. Piatek, A. S., Tyagi, S., Pol, A. C., Telenti, A., Miller, L. P. *et al.* (1998) *Nat Biotechnol* 16(4):359-63.
4. Tyagi, S., Bratu, D. P. and Kramer, F. R. (1998) *Nat Biotechnol* 16(1):49-53.
5. Tyagi, S. and Kramer, F. R. (1996) *Nat Biotechnol* 14(3):303-8.
6. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology (N Y)* 11(9):1026-30.
7. Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. (1991) *Proc Natl Acad Sci U S A* 88(16):7276-80.
8. Edwards, M. and Gibbs, R. (1995). Multiplex PCR. In *PCR Primer: A Laboratory Manual*,C. W. Dieffenbach and G. S. Dveksler (Eds.), pp. 157-171. Cold Spring Harbor Laboratory Press, Plainview, NY.
9. Chamberlain, J. S. and Chamberlain, J. R. (1994). Optimization of Multiplex PCR. In *The Polymerase Chain Reaction*,K. B. Mullis, F. Ferré and R. A. Gibbs (Eds.), pp. 38-46. Birkhauser Boston Press, Boston.
10. McBride, L., Livak, K., Lucero, M., Goodsaid, F., Carlson, D. *et al.* (1998). Quantitative PCR Technology. In *Gene Quantification*,F. Ferré (Ed.), pp. 97-110. Birkhauser Boston Press, Boston.
11. Marras, S. A., Kramer, F. R. and Tyagi, S. (1999) *Genet Anal* 14(5-6):151-6.
12. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

BRILLIANT MULTIPLEX QPCR MASTER MIX

Catalog #600553

QUICK-REFERENCE PROTOCOL

1. If the passive reference dye will be included in the reaction (optional), dilute 1:500 (for the Mx4000, Mx3000P, and Mx3005P instruments). For other instruments, use the guidelines in *Reference Dye* in *Preprotocol Considerations* for passive reference dye optimization. **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 multiplex 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.*

3. Prepare the experimental reaction by adding the following components *in order*:

   ## Experimental Reaction

   Nuclease-free PCR-grade water 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 diluted reference dye (**optional**)

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

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

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

PCR Programs for the Agilent Mx3000P, Mx3005P, and Mx4000 Instruments

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

Two-Step PCR Cycling Protocol

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

Three-Step PCR Cycling Protocol

Cycles	Duration of cycle	Temperature
1	10 minutes[a]	95°C
40	15 seconds	95°C
	1.0 minute[b]	55–60°C[c]
	30 seconds	72°C

[a] The 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.

PCR Programs for Other Spectrofluorometric Thermal Cyclers

Place the reactions in the spectrofluorometric thermal cycler and run the recommended two- or three-step cycling program, as recommended by the instrument manufacturer.