Brilliant III Ultra-Fast QPCR Master Mix

Instruction Manual

Catalog #600880 (single kit)
#600881 (10-pack kit)
Revision D0

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600880-12

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Brilliant III Ultra-Fast QPCR Master Mix

Contents

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MATERIALS PROVIDED

Catalog #600880 (single kit), #600881 (10-pack kit)

Materials Provided	Quantity[a,b]
2× Brilliant III Ultra-Fast QPCR Master Mix	2 × 2 ml
Reference dye[c], 1 mM	100 µl

[a] Sufficient PCR reagents are provided for four hundred, 20-µl reactions.

[b] Quantities listed are for a single kit. For 10-pack kits, each item is provided at 10 times the listed quantity.

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

STORAGE CONDITIONS

All Components: Store at –20°C upon receipt. After thawing, the 2× master 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

NOTICE TO PURCHASER: LIMITED LICENSE
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Revision D0

INTRODUCTION

The Brilliant III Ultra-Fast QPCR Master Mix is a single-tube reagent designed for performing accelerated quantitative PCR amplifications with fluorescent TaqMan® probes on the ABI StepOnePlus and Bio-Rad CFX96 real-time PCR instruments and other fast-cycling systems (such as the ABI 7900HT and 7500 Fast systems). The master mix includes two key components that enable it to perform optimally under fast cycling conditions:

- A mutated form of *Taq* DNA polymerase that has been specifically engineered for faster replication
- An improved chemical hot start mechanism that promotes faster hot start release to improve amplification specificity while keeping the run time of the PCR protocol to a minimum

The Brilliant III Ultra-Fast QPCR master mix has been successfully used with TaqMan probes to amplify and detect a variety of DNA targets, including cDNA, genomic DNA and plasmid DNA.

The 2× master mix contains the mutant *Taq* DNA polymerase, dNTPs, Mg^{2+} and a buffer specially formulated for fast cycling. 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 multiple platforms.

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).[1] 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.[1] 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 1 shows an ABI StepOnePlus instrument amplification plot with Ct determination (top panel) and standard curve (bottom panel). In this experiment, the beta-2-microglobulin gene was amplified and detected using a TaqMan probe and the Brilliant III Ultra-Fast QPCR master mix.

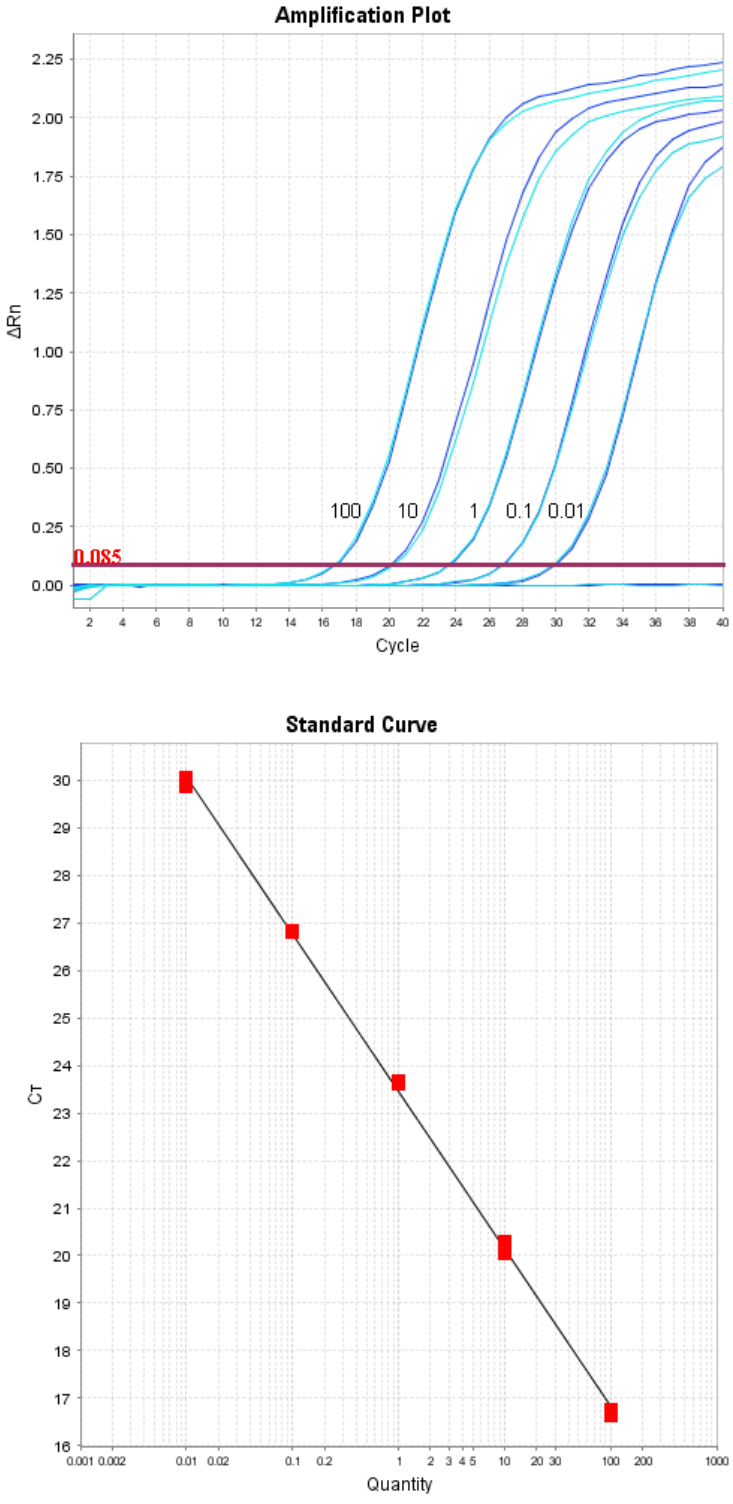

Figure 1 *Top panel*: StepOnePlus instrument amplification plot using a TaqMan® probe. A serial dilution of cDNA template was added to each reaction and reactions were performed in duplicate. The amount of cDNA template added per reaction (in ng) is indicated to the left of each amplification curve. 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 100.1% and an R-squared 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 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 150 to 600 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.

Magnesium Chloride

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

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 not required when using the Bio-Rad CFX96 real-time PCR system, with other instruments (including the ABI StepOnePlus instrument) 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 StepOnePlus or 7900HT Fast instrument, dilute the dye 1:50 for a final concentration of 300 nM in the reactions. For the Agilent Mx instruments or the ABI 7500 Fast instrument, dilute the dye 1:500 for a final concentration of 30 nM. The Bio-Rad CFX96, the Roche LightCycler® 480 and the QIAGEN Rotor-Gene Q instruments do not require the use of the reference dye.

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 III Ultra-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.[2] Multiplex PCR in which two or more sequences are amplified simultaneously can often be performed using the conditions for amplification of a single sequence.[3] The Brilliant III Ultra-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.[4] 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.[4]

PROTOCOL

Preparing the Reactions

Notes *Once the tube containing the 2× 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 return to –20°C for long term storage.*

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

1. If using the reference dye, dilute the provided dye using nuclease-free PCR-grade H_2O. **Keep all solutions containing the reference dye protected from light.**

   - For the ABI StepOnePlus instrument or the ABI 7900HT Fast instrument, dilute the dye **1:50** (for a final concentration of 300 nM in the reactions).

   - For the Agilent AriaMx, Mx3000P, or Mx3005P instrument or the ABI 7500 Fast instrument, dilute the dye **1:500** (for a final concentration of 30 nM in the reactions).

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

   ### Reagent Mixture

   Nuclease-free PCR-grade H_2O to adjust the final volume to 20 µl (including experimental DNA)
   10 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)
   0.3 µl of the **diluted** reference dye (optional)

3. Gently mix the reagents without creating bubbles (do not vortex), then distribute the mixture to individual PCR reaction tubes.

4. Add *x* µl of experimental DNA to each reaction to bring the final reaction volume to 20 µl. The table below lists a suggested quantity range for different DNA templates.

DNA	Quantity per reaction
Genomic DNA	5 pg–100 ng
cDNA	0.1 pg–100 ng*

* Refers to RNA input amount during cDNA synthesis

5. Gently mix the reactions without creating bubbles (do not vortex), then centrifuge the reactions briefly.

   Note *Bubbles interfere with fluorescence detection.*

PCR Cycling Programs

6. Place the reactions in the instrument. Based on the instrument you are using, select the appropriate PCR program from the tables below. Set the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

Note *For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system. Genomic targets generally require longer denaturation and annealing/extension times than low-complexity targets (e.g. cDNA and plasmid DNA).*

Agilent AriaMx

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

Agilent Mx3000P and Mx3005P

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5–20 seconds	95°C
	20 seconds	60°C

ABI 7500 Fast

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	12 seconds	95°C
	15 seconds	60°C

ABI 7900HT Fast

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5 seconds	95°C
	15 seconds	60°C

ABI StepOnePlus

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

Bio-Rad CFX96

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

QIAGEN Rotor-Gene Q

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5–20 seconds	95°C
	10–20 seconds	60°C

Roche LightCycler® 480

Cycles	Duration of cycle	Temperature
1	3 minutes	95°C
40	5 seconds	95°C
	10 seconds	60°C

TROUBLESHOOTING

Observation	Suggestion
There is a low increase in fluorescence with cycling or none at all	The efficiency of PCR is low because the PCR product is too long. Design the primers so that the PCR product is <150 bp in length.
	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 performing a nuclease digestion to ensure it is unquenching as expected.
	Redesign the probe using Primer Express or other software. Design a probe that performs well in reactions containing 5.5 mM $MgCl_2$.
	The DNA polymerase was not activated. Ensure that the 3-minute initial incubation at 95°C was performed as part of the cycling parameters.
	The DNA polymerase was activated for more than 3 minutes. Ensure that the initial 95°C incubation was not longer than 3 minutes.
	The reaction is not optimized and 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 5 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.

REFERENCES

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2. 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.
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4. 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.
5. Kwok, S. and Higuchi, R. (1989) *Nature* 339(6221):237-8.

ENDNOTES

LightCycler® is a registered trademark of Roche.
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 III ULTRA-FAST QPCR MASTER MIX

Catalog #600880, #600881

QUICK-REFERENCE PROTOCOL

Prior to setting up the reactions, thaw the 2× 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.

1. If using the reference dye, dilute the provided dye with nuclease-free PCR-grade H_2O. For the ABI StepOnePlus instrument or the ABI 7900HT Fast instrument, dilute the dye 1:50 (for a final concentration of 300 nM in the reactions). For an Agilent Mx instrument or the ABI 7500 Fast instrument, dilute the dye 1:500 (for a final concentration of 30 nM in the reactions). Keep all solutions containing the reference dye protected from light.

2. Prepare the experimental reactions by adding the following components *in order*. Prepare a single reagent mixture for multiple reactions using multiples of each component listed below.

   Reagent Mixture

   Nuclease-free PCR-grade H_2O to bring the final volume to 20 µl (including DNA)
   10 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)
   0.3 µl of diluted reference dye (optional)

3. Gently mix without creating bubbles (bubbles interfere with fluorescence detection; do not vortex), then distribute the mixture to individual PCR reaction tubes.

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

5. Gently mix the reactions without creating bubbles, then centrifuge the reactions briefly.

6. Place the reactions in the instrument. Based on the instrument you are using, select the appropriate PCR program from the tables below. Set the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

Note *For optimal performance, the durations of the denaturation and annealing/extension steps may need to be adjusted for each probe/target system. Genomic targets generally require longer denaturation and annealing/extension times than low-complexity targets (e.g. cDNA and plasmid DNA).*

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QIAGEN Rotor-Gene Q

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Roche LightCycler® 480

Cycles	Duration of cycle	Temperature
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