Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix

Instruction Manual

Catalog #600882 (single kit)
#600883 (10-pack kit)
Revision E.0

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

600882-12

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MATERIALS PROVIDED

Catalog #600882 (single kit), #600883 (10-pack kit)

Materials Provided	Quantity[a,b]
2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix[c, d]	2 × 2 ml
Reference dye[d], 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 master mix contains nucleotide mix GATC.

[d] The master mix and reference dye are 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 SYBR Green master mix and the reference dye are light sensitive and should be kept away from light whenever possible.*

ADDITIONAL MATERIALS REQUIRED

Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

Revision E.0

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INTRODUCTION

The Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix is a single-tube reagent designed for performing accelerated quantitative PCR amplifications 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 2× master mix contains the mutant *Taq* DNA polymerase, dNTPs, Mg^{2+}, a buffer specially formulated for fast cycling, and the double-stranded DNA-binding dye SYBR Green I for detection. 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 Using SYBR® Green

When fluorescence signal from a SYBR Green-based PCR reaction is monitored in real-time, the results can be displayed as an amplification plot which reflects the change in fluorescence during cycling. 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. In the amplification plot in Figure 1, for example, the Ct of each reaction is the cycle number at which the plot crosses the threshold line. The threshold cycle has been shown to be inversely proportional to the log of the initial copy number.[2] The more template that is initially present, the fewer the number of cycles it takes to get to a point where the fluorescence signal is detectable above background. Quantitative information based on threshold cycle is more accurate than information based on endpoint determinations as it is based on measurements taken during the exponential phase of PCR amplification when the PCR efficiency has yet to be influenced by limiting reagents, small differences in reaction components, or cycling conditions.

In Figure 1, the Brilliant III Ultra-Fast SYBR Green QPCR master mix was used in reactions containing serially diluted cDNA template (0.5 pg – 50 ng) and a no-template control reaction (NTC) to amplify the GAPDH target. In the amplification plot (top panel) the reactions containing template show a significant increase in fluorescence with Ct values ranging from 17 to 33. The NTC reaction has no Ct because the amplification plot does not cross the threshold. The Ct values obtained in the amplification plot are used to generate the standard curve in the bottom left panel. In the dissociation/melt curve (bottom right panel), PCR samples were subjected to a stepwise increase in temperature from 60°C to 95°C with fluorescence measurements taken throughout this range. The first derivative of fluorescence is then plotted versus temperature. As the temperature increases, the amplification

products in each tube melt according to their composition. In Figure 1, the melt curve shows fluorescence peaks centered at 83.76°C, which correspond to the desired product in the reactions. If primer-dimer or nonspecific products had been made during the amplification step, they would generally melt at a lower temperature (defined as the Tm) than the desired products.

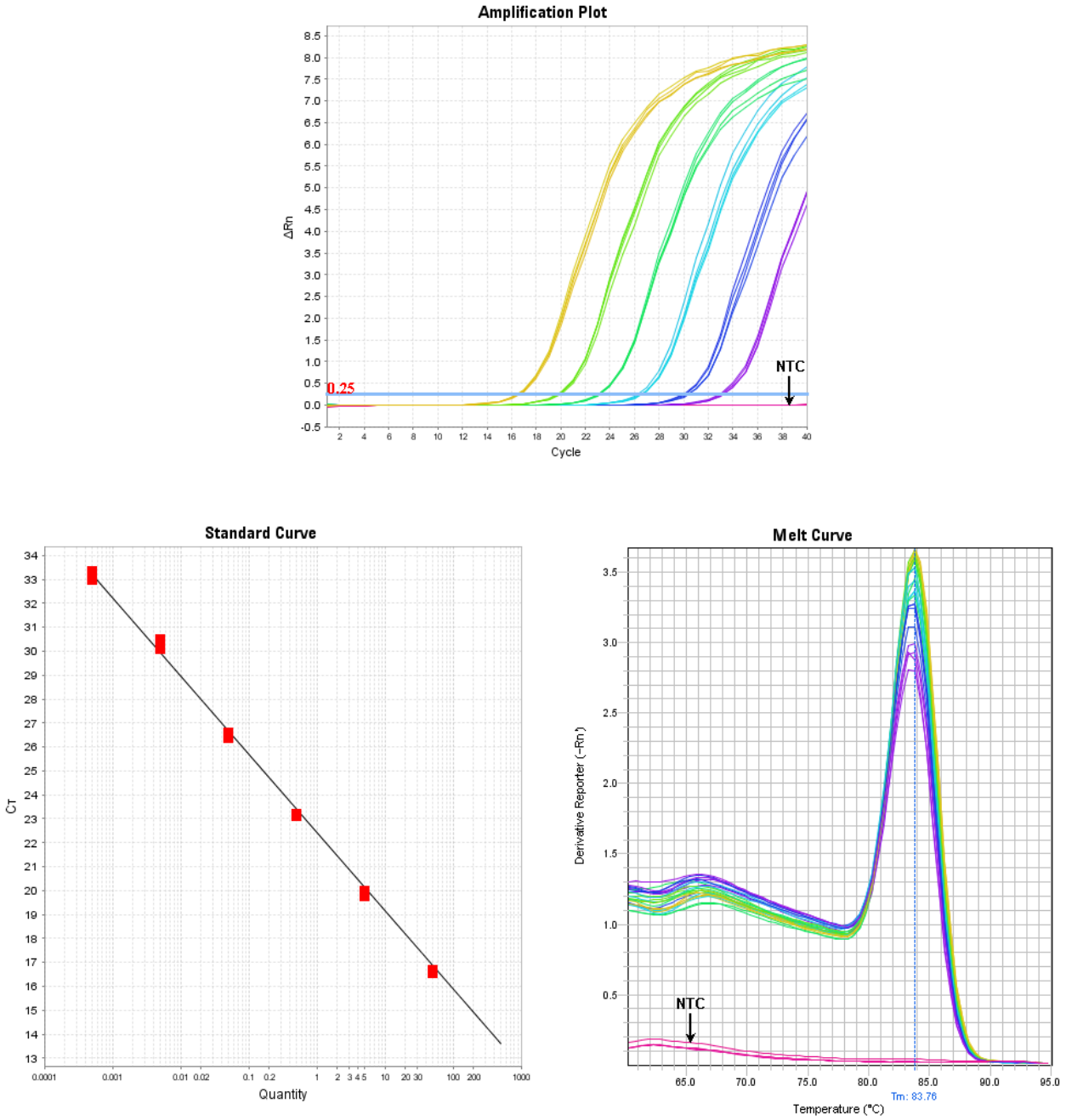

FIGURE 1 *TOP PANEL*: STEPONEPLUS INSTRUMENT AMPLIFICATION PLOT. A SERIAL DILUTION OF CDNA TEMPLATE WAS ADDED (IN QUADRUPLICATE) TO EACH REACTION. THE FLUORESCENCE VALUE USED TO DETERMINE CT (THE THRESHOLD LINE) IS SHOWN AS A SOLID LINE. *BOTTOM LEFT PANEL*: STANDARD CURVE GENERATED FROM AMPLIFICATION PLOT. AN AMPLIFICATION EFFICIENCY OF 102.56% AND AN R-SQUARED VALUE OF 0.998 WERE OBTAINED. *BOTTOM RIGHT PANEL*: MELT CURVE GENERATED WHEN AMPLIFIED PRODUCTS WERE SUBJECTED TO DISSOCIATION ANALYSIS. THE FLUORESCENCE PEAKS CORRESPONDING TO THE AMPLICON ARE CENTERED AROUND 83.76°C.

PREPROTOCOL CONSIDERATIONS

PCR Primers

It is critical in SYBR Green-based QPCR to minimize the formation of non-specific amplification products. This issue becomes more prominent at low target concentrations. Therefore, to maximize the sensitivity of the assay, it is necessary to use the lowest concentration of primers possible without compromising the efficiency of PCR. It is important to consider both the relative concentrations of forward and reverse primers and the total primer concentration. The optimal concentration of the upstream and downstream PCR primers is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration, with minimal or no formation of primer-dimer. This concentration should be determined empirically. Generally, primer concentrations in the range of 200–500 nM are satisfactory.

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.

* 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.

Magnesium Chloride

The optimal $MgCl_2$ concentration promotes maximal amplification of the specific target amplicon with minimal nonspecific products and primer-dimer formation. High levels of the Mg^{2+} ion tend to favor the formation of nonspecific dsDNA, including primer-dimers. Therefore, when a SYBR Green-based QPCR assay is being optimized, the $MgCl_2$ levels should be as low as possible without compromising the efficiency of amplification of the specific target (typically between 1.5 and 2.5 mM $MgCl_2$). The Brilliant III Ultra-Fast SYBR Green QPCR master mix contains $MgCl_2$ at a concentration of 2.5 mM (in the 1× solution), which is suitable for most targets. The concentration may be increased, if desired, by adding a small amount of a concentrated $MgCl_2$ solution to the 1× experimental reaction at the time of setup.

Data Acquisition with a Spectrofluorometric Thermal Cycler

The instrument should be set to collect SYBR Green I data in real-time at the annealing/extension step of each cycle. How this is accomplished will depend on the software that commands the particular instrument you are using. Consult the manufacturer's instruction manual for the instrument and software version you are using.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction.[2] Because SYBR Green I dye fluoresces in the presence of any dsDNA, multiplexing with the Brilliant III Ultra-Fast SYBR Green QPCR master mix is not recommended.

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.

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.*

SYBR Green dye is light-sensitive; solutions containing the master mix should be protected from light whenever possible.

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.

Reagent Mixture

Nuclease-free PCR-grade water to adjust the final volume to 20 µl (including experimental DNA)
10 µl of 2× SYBR Green QPCR master mix
x µl of upstream primer (200–500 nM final concentration)
x µl of downstream primer (200–500 nM final concentration)
0.3 µl of diluted reference dye (optional)

3. Gently mix 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–50 ng
cDNA	0.5 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 target. 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
	5–10 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	5 seconds	95°C
	12 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
	5–10 seconds	60°C

QIAGEN Rotor-Gene Q

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

Dissociation Programs

7. For your specific instrument, follow the manufacturer's guidelines for generating dissociation curves.

TROUBLESHOOTING

Observation	Suggestion(s)
No (or little) increase in fluorescence with cycling	Optimize the primer concentration.
	The target is highly GC-rich. Raise the denaturation temperature to 98°C or titrate DMSO into the reactions in 1% increments.
	Ensure that the correct concentration and amount of template was used and that the template sample is of good quality. If unsure, make new serial dilutions of template before repeating PCR. It may also be possible to check for PCR inhibitors by adding this target into an assay that is known to work.
	Use a sufficient number of cycles in the PCR reaction.
	Gel analyze PCR product to determine if there was successful amplification.
	Ensure the correct dilution of reference dye was used.
	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 $MgCl_2$ concentration is not optimal. The $MgCl_2$ concentration in the 1× Brilliant III Ultra-Fast SYBR Green QPCR master mix is 2.5 mM. It is possible to add small amounts of concentrated $MgCl_2$ to the experimental reactions to increase the $MgCl_2$ concentration, if desired.
	Target length may be too long for sufficient amplification with fast cycling. Design the primers so that the PCR product is <300 bp in length.
There is a large abundance of primer-dimer and nonspecific PCR products	Reduce primer concentrations or re-design primers.

REFERENCES

1. Higuchi, R., Fockler, C., Dollinger, G. and Watson, R. (1993) *Biotechnology (N Y)* 11(9):1026-30.
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.

ENDNOTES

LightCycler® is a registered trademark of Roche.
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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 SYBR® GREEN QPCR MASTER MIX

Catalog #600882, #600883

QUICK-REFERENCE PROTOCOL

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

SYBR Green I dye (present in the master mix) is light-sensitive; solutions containing the master mix should be protected from light whenever possible.

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 adjust the final volume to 20 µl (including experimental DNA)
   10 µl of 2× SYBR Green QPCR master mix
   x µl of upstream primer (200–500 nM final concentration is recommended)
   x µl of downstream primer (200–500 nM final concentration is recommended)
   0.3 µl of **diluted** reference dye from step 1 (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 experimental gDNA, cDNA, or plasmid DNA to each reaction to bring the final reaction volume to 20 µl.

5. Gently mix the reactions without creating bubbles (do not vortex), 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 target. 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
	5–10 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	5 seconds	95°C
	12 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
	5–10 seconds	60°C

QIAGEN Rotor-Gene Q

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

7. For your specific instrument, follow the manufacturer's guidelines for generating dissociation curves.