Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX

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600903

Protocol

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For Research Use Only. Not for Use in Diagnostic Procedures.

Agilent Technologies

Notices

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Technical Support

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Endnotes

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In this Guide...

This document describes how to use the Agilent Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX to perform quantitative PCR amplifications with an accelerated cycling protocol.

Contents

1 Before You Begin

This chapter provides important information on getting started with a QPCR experiment using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX.

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Kit contents

Table 1 shows the materials provided with the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX.

Sufficient reagents are provided for four hundred 20-µL QPCR reactions.

Table 1 Kit contents

Material	Catalog #	Quantity
2× Brilliant III SYBR Green QPCR Master Mix with Low ROX*	600892	2 × 2 mL (400 20-µL reactions)
	600903	20 × 2 mL (4000 20-µL reactions)

* The concentration of ROX dye in the final reactions is 30 nM.

Storage conditions for the master mix

Store at –20°C upon receipt. After thawing, the master mix may be stored at 4°C for one month or returned to –20°C for long term storage.

The master mix is light sensitive and should be kept away from light whenever possible.

Required reagents and equipment

Table 2 contains a list of reagents and equipment that are required for the QPCR protocol.

Table 2 Required Reagents and Equipment

Description
Spectrofluorometric thermal cycler
Nuclease-free PCR-grade water

Overview of the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with Low ROX

The Brilliant III Ultra-Fast SYBR Green QPCR Master Mix with Low ROX is a single-tube reagent designed for performing accelerated quantitative PCR amplifications using SYBR Green I dye for amplicon detection. The master mix includes 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

Reactions prepared with the low ROX master mix contain 30 nM of ROX reference dye. This concentration of ROX dye is suitable for Agilent's real-time PCR instruments (AriaMx, Mx3000P, and Mx3005P) and the ABI 7500 Fast real-time PCR instrument from Applied Biosystems.

The 2× master mix also contains SYBR Green I dye, dNTPs (nucleotide mix GATC), Mg^{2+}, and a buffer specially formulated for fast cycling.

2 Procedures

This chapter provides guidelines and instructions on how to perform QPCR with the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix with Low ROX.

Preprotocol Considerations

PCR Primers

It is critical in SYBR Green-based QPCR to minimize the formation of nonspecific amplification products. This issue becomes more prominent at low target concentrations. Therefore, to maximize the sensitivity of the assay, use the lowest concentration of primers possible without compromising the efficiency of PCR. Take into consideration 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.

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 optimizing a SYBR Green-based QPCR assay, keep the $MgCl_2$ levels 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. If desired, you can increase the concentration 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

Set the instrument 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. 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.

QPCR Protocol

Prepare the reactions

NOTE

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. Avoid multiple freeze-thaw cycles.

Set up a no-template control reaction to screen for contamination of reagents or false amplification.

Keep all solutions containing the master mix protected from light as much as possible.

1 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 in Table 3.

Table 3 QPCR reagent mixture

Component	Volume per reaction
Nuclease-free PCR-grade water	X µL (enough to yield a final reaction volume of 20 µL, including experimental DNA)
2× Brilliant III SYBR Green QPCR Master Mix with Low ROX	10 µL
Upstream primer	X µL (200–500 nM final concentration)
Downstream primer	X µL (200–500 nM final concentration)

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

3 Add x µL of experimental DNA to each reaction. Table 4 lists a suggested quantity range for different DNA templates.

Table 4 Quantity of template DNA per reaction

DNA	Quantity per reaction
Genomic DNA	5 pg – 50 ng
cDNA	0.5 pg – 100 ng*

* Refers to RNA input amount during cDNA synthesis

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

Bubbles interfere with fluorescence detection

Run the PCR cycling program

- Place the reactions in the instrument. Run the cycling program shown below that is appropriate for your instrument. Set the intrument 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).

Table 5 PCR program for the Agilent AriaMx

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	3 minutes*
2	40	95°C	5 seconds
		60°C	5–10 seconds

Table 6 PCR program for the Agilent Mx3000P or Mx3005P

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	3 minutes*
2	40	95°C	5–20 seconds
		60°C	20 seconds

Table 7 PCR program for the ABI 7500 Fast

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	3 minutes*
2	40	95°C	5 seconds
		60°C	12 seconds

* Initial 3-minute incubation is required to activate the DNA polymerase.

Generate a dissociation curve

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

3 Troubleshooting

If the increase in fluorescence with cycling is low or nonexistant

The target length is too long for sufficient amplification with fast cycling.

✔ Design the primers so that the PCR product is <300 bp in length.

The DNA polymerase is not functioning optimally.

✔ Make sure that the 3-minute initial incubation at 95°C was performed as part of the cycling program.

✔ Make sure that the initial 95°C incubation was not longer than 3 minutes.

The reaction is not optimized and insufficient product is formed.

✔ Test for formation of enough specific product by gel electrophoresis.

✔ Optimize the primer concentration.

✔ The $MgCl_2$ concentration in the 1× master mix is 2.5 mM. Try adding small amounts of concentrated $MgCl_2$ (not included in this kit) to the experimental reactions to increase the $MgCl_2$ concentration.

The concentration or quality of the template is not optimal.

✔ Make sure 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.

✔ Check for PCR inhibitors in the template by adding this target into an assay this is known to work.

The target is highly GC-rich.

✔ Raise the denaturation temperature to 98°C or titrate DMSO into the reactions in 1% increments.

If the level of primer-dimer and nonspecific products is high

The primers are hybridizing to nonspecific sites.

✔ Reduce the primer concentrations.

✔ Design new primers.

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