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

Catalog #
600892
600903

Protocol
Version C0, January 2015

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Agilent Technologies
Notices

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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.

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.

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.

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Before You Begin

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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

<table>
<thead>
<tr>
<th>Material</th>
<th>Catalog #</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2× Brilliant III SYBR Green QPCR Master Mix</td>
<td>600892</td>
<td>2 × 2 mL (400 20-μL reactions)</td>
</tr>
<tr>
<td>with Low ROX*</td>
<td>600903</td>
<td>20 × 2 mL (4000 20-μL reactions)</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrofluorometric thermal cycler</td>
</tr>
<tr>
<td>Nuclease-free PCR-grade water</td>
</tr>
</tbody>
</table>
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), Mg²⁺, and a buffer specially formulated for fast cycling.
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 MgCl₂ concentration promotes maximal amplification of the specific target amplicon with minimal nonspecific products and primer-dimer formation. High levels of the Mg²⁺ ion tend to favor the formation of nonspecific dsDNA, including primer-dimers. Therefore, when optimizing a SYBR Green-based QPCR assay, keep the MgCl₂ levels as low as possible without compromising the efficiency of amplification of the specific target (typically between 1.5 and 2.5 mM MgCl₂). The Brilliant III Ultra-Fast SYBR Green QPCR master mix contains MgCl₂ 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 MgCl₂ 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

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

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.
Prepare the reactions

**Table 4** Quantity of template DNA per reaction

<table>
<thead>
<tr>
<th>DNA</th>
<th>Quantity per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>5 pg – 50 ng</td>
</tr>
<tr>
<td>cDNA</td>
<td>0.5 pg – 100 ng*</td>
</tr>
</tbody>
</table>

* 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
2 Procedures

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 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).

Table 5  PCR program for the Agilent AriaMx

<table>
<thead>
<tr>
<th>Segment</th>
<th>Number of Cycles</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>3 minutes*</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>95°C</td>
<td>5 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>5–10 seconds</td>
</tr>
</tbody>
</table>

Table 6  PCR program for the Agilent Mx3000P or Mx3005P

<table>
<thead>
<tr>
<th>Segment</th>
<th>Number of Cycles</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>3 minutes*</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>95°C</td>
<td>5–20 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>20 seconds</td>
</tr>
</tbody>
</table>

Table 7  PCR program for the ABI 7500 Fast

<table>
<thead>
<tr>
<th>Segment</th>
<th>Number of Cycles</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>3 minutes*</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>95°C</td>
<td>5 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60°C</td>
<td>12 seconds</td>
</tr>
</tbody>
</table>

* 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.
2 Procedures
Generate a dissociation curve
3 Troubleshooting

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If the increase in fluorescence with cycling is low or nonexistent

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\textsubscript{2} concentration in the 1× master mix is 2.5 mM. Try adding small amounts of concentrated MgCl\textsubscript{2} (not included in this kit) to the experimental reactions to increase the MgCl\textsubscript{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.
Troubleshooting

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

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.