Brilliant III Ultra-Fast QPCR Master Mix with Low ROX

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Protocol

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

Agilent Technologies

Notices

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In this Guide...

This document describes how to use the Agilent Brilliant III Ultra-Fast QPCR Master Mix with Low ROX to perform probe-based 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 QPCR Master Mix with Low ROX.

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Kit contents

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

Table 1 Kit contents

Material	Catalog #	Quantity
2× Brilliant III QPCR Master Mix with Low ROX*	600890	2 × 2 mL (400 20-µL reactions)
	600898	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 QPCR Master Mix with Low ROX

The Brilliant III Ultra-Fast QPCR Master Mix with Low ROX is a single-tube reagent designed for performing accelerated quantitative PCR amplifications with fluorescent TaqMan® probes. 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 dNTPs, Mg^{2+}, and a buffer specially formulated for fast cycling.

The Brilliant III Ultra-Fast QPCR Master Mix with Low ROX has been successfully used to amplify and detect a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA.

2 Procedures

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

Preprotocol Considerations

Magnesium Chloride

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

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 Piers

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 in increments of 100 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.

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 re-amplification 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.

Multiplex PCR

Multiplex PCR is the amplification of more than one target in a single polymerase chain reaction. The Brilliant III Ultra-Fast QPCR master mix has been successfully used to amplify two targets in a multiplex reaction without re-optimizing 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, try to minimize competition between concurrent amplifications for common reagents by determining the limiting primer concentrations for the more abundant target.* Also consider optimizing the other reaction components. The number of fluorophores in each tube can influence the analysis.

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.*

* McBride, L., Livak, K., Lucero, M., Goodsaid, F., Carlson, D. *et al.* (1998) Quantitative PCR Technology. In *Gene Quantification*, F. Ferre (Ed.), pp. 97-110. Birkhauser Boston Press, Boston.

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 QPCR Master Mix with Low ROX	10 µL
Experimental fluorescent probe	X µL (optimized concentration)
Upstream primer	X µL (optimized concentration)
Downstream primer	X µL (optimized 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 – 100 ng
cDNA	0.1 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 probe/target system. 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	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	12 seconds
		60°C	15 seconds

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

3
Troubleshooting

If the increase in fluorescence with cycling is low or nonexistant

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.

If performing multiplex PCR, one of the reagents may be at an insufficient concentration.

✔ Add a small amount of concentrated $MgCl_2$ (not provided with this kit) to the 1× experimental reaction at the time of set up.

✔ Supplement the reactions with additional polymerase and dNTPs (not provided with this kit).

The probe has a nonfunctioning fluorophore.

✔ Make sure that the fluorophore functions by performing a nuclease digestion to ensure it is unquenching as expected.

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.

The probe is not well designed for the reaction conditions.

✔ Redesign the probe using Primer Express or other software.

✔ Design a probe that performs well in reactions containing 5.5 mM $MgCl_2$.

If there is increased fluorescence in no-template control reactions

The reaction has been contaminated.

✔ Follow the procedures outlined in the reference at the bottom of page 13.

✔ Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See "Preventing Template Cross-Contamination" on page 12.

If the Ct in the no-template control sample is less than the total number of cycles but the amplification plot is flat

The background level of fluorescence is varying from cycle to cycle.

✔ Review the amplification plot and, if appropriate, adjust the threshold accordingly.

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