

Brilliant III Ultra-Fast QPCR Master Mix with High ROX

**Catalog #
600888
600899**

Protocol

Version B0, July 2014

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



Agilent Technologies

Notices

© Agilent Technologies, Inc. 2014

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Manual Part Number

600888-12

Edition

Version B0, July 2014

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Rd
Santa Clara, CA 95051 USA

Technical Support

For technical product support, contact Agilent at (800) 227-9770 or techservices@agilent.com

Warranty

The material contained in this document is provided “as is,” and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A **WARNING** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a **WARNING** notice until the indicated conditions are fully understood and met.

In this Guide...

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

2 Procedures

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

3 Troubleshooting

1	Before You Begin	5
	Notice to Purchaser: Limited License	6
	Kit contents	6
	Storage conditions for the master mix	7
	Required reagents and equipment	7
	Overview of the Brilliant III Ultra-Fast QPCR Master Mix with High ROX	8
2	Procedures	9
	Preprotocol Considerations	10
	Magnesium Chloride	10
	Probe Design	10
	Optimal Concentrations for Experimental Probes and Primers	10
	Preventing Template Cross-Contamination	11
	Multiplex PCR	12
	QPCR Protocol	13
	Prepare the reactions	13
	Run the PCR cycling program	14
3	Troubleshooting	15
	If the increase in fluorescence with cycling is low or nonexistent	16
	If there is increased fluorescence in no-template control reactions	17
	If the Ct in the no-template control sample is less than the total number of cycles but the amplification plot is flat	17



1 Before You Begin

Notice to Purchaser: Limited License	6
Kit contents	6
Storage conditions for the master mix	7
Required reagents and equipment	7
Overview of the Brilliant III Ultra-Fast QPCR Master Mix with High ROX	8

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



Notice to Purchaser: Limited License

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 6,258,569, 6,171,785, 6,127,155, 6,030,787, 5,994,056, 5,876,930, 5,804,375, 5,789,224, 5,773,258 (claims 1 and 6 only), 5,723,591, 5,677,152 (claims 1 to 23 only), 5,618,711, 5,538,848, and claims outside the US corresponding to expired US Patent No. 5,079,352. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

Kit contents

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

Table 1 Kit contents

Material	Catalog #	Quantity
2× Brilliant III QPCR Master Mix with High ROX*	600888	2 × 2 mL (400 20-μL reactions)
	600899	20 × 2 mL (4000 20-μL reactions)

* The concentration of ROX dye in the final reactions is 300 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 High ROX

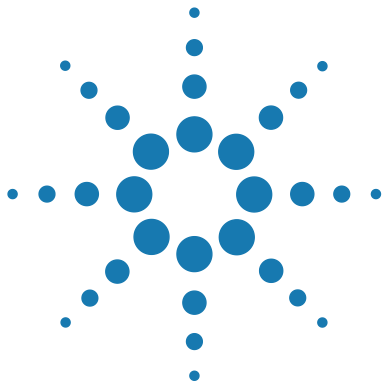
The Brilliant III Ultra-Fast QPCR Master Mix with High ROX is a single-tube reagent designed for performing accelerated quantitative PCR amplifications with fluorescent TaqMan[®] probes. The master mix includes components that enable it to perform optimally under fast cycling conditions:

- 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 high ROX master mix contain 300 nM of ROX reference dye. This concentration of ROX dye is suitable for the ABI 7900HT Fast and StepOnePlus real-time PCR instruments from Applied Biosystems.

The 2× master mix also contains dNTPs, Mg²⁺, and a buffer specially formulated for fast cycling.

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



2 Procedures

Preprotocol Considerations	10
Magnesium Chloride	10
Probe Design	10
Optimal Concentrations for Experimental Probes and Primers	10
Preventing Template Cross-Contamination	11
Multiplex PCR	12
QPCR Protocol	13
Prepare the reactions	13
Run the PCR cycling program	14

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

- 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 instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step.

Table 5 PCR program for the ABI 7900HT Fast

Segment	Number of Cycles	Temperature	Duration
1	1	95°C	3 minutes*
2	40	95°C	5 seconds
		60°C	15 seconds

Table 6 PCR program for the ABI StepOnePlus

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

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



3 Troubleshooting

If the increase in fluorescence with cycling is low or nonexistent [16](#)

If there is increased fluorescence in no-template control reactions [17](#)

If the Ct in the no-template control sample is less than the total number of cycles but the amplification plot is flat [17](#)



If the increase in fluorescence with cycling is low or nonexistent

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 12](#).
- ✓ Perform decontamination during amplification by including uracil-N-glycosylase (UNG) in the PCR reaction mix. See “[Preventing Template Cross-Contamination](#)” on page 11.

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.

www.agilent.com

In this book

This document describes how to use the Agilent Brilliant III Ultra-Fast QPCR Master Mix with High ROX to perform probe-based quantitative PCR amplifications with an accelerated cycling protocol.

© Agilent Technologies, Inc. 2014

Version B0, July 2014



600888-12