

SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR SuperMix with BSA

Cat. no. 11750-100
Cat. no. 11750-500

Size: 96 reactions
Size: 480 reactions
Store at -20°C

Description

SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR SuperMix with BSA is a ready-to-use reaction mix for one-step, real-time quantitative RT-PCR (qRT-PCR) that has been specifically formulated for use with the Roche LightCycler®. The SuperMix combines the high-temperature reverse transcription capability of SuperScript™ III Reverse Transcriptase (RT), the automatic hot-start PCR provided by Platinum® *Taq* DNA Polymerase, and the fluorescent binding dye SYBR® Green I for optimal specificity, consistency, and efficiency. Both cDNA synthesis and PCR are performed in the same tube using gene-specific primers and RNA target(s) from either total RNA or mRNA. All components necessary for qRT-PCR are combined in the tube, and reverse transcription is directly followed by PCR cycling without additional handling.

The SuperMix formulation enables highly sensitive detection down to 0.1 pg of total RNA, and has a broad quantification range from 1 pg to 1 µg of total RNA. It is provided at 2.5X concentration, and includes SuperScript™ III RT; Platinum® *Taq* DNA Polymerase; SYBR® Green I dye; 7.5 mM MgCl₂; dNTPs; ultrapure, nonacetylated bovine serum albumin (BSA); a proprietary buffer system; and stabilizers.

SuperScript™ III Reverse Transcriptase is a version of M-MLV RT that has been engineered to reduce RNase H activity and provide increased thermal stability (1, 2). The enzyme can be used to synthesize cDNA at a temperature range of 42–60°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases. Because SuperScript™ III RT is not significantly inhibited by ribosomal and transfer RNA, it can be used to synthesize cDNA from total RNA.

Platinum® *Taq* DNA polymerase is recombinant *Taq* DNA polymerase complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures (3, 4, 5). Activity is restored after the denaturation step in PCR cycling, providing an automatic “hot start” in PCR for increased sensitivity, specificity, and yield.

SYBR® Green I is a fluorescent dye that binds directly to double-stranded DNA (dsDNA). In qPCR, as dsDNA accumulates, the dye generates a signal that is proportional to the DNA concentration and that can be detected using real-time qPCR instruments (2, 3). SYBR® Green I in this formulation can detect as few as 10 copies of a target gene in as little as 0.1 pg of total RNA, has a wide dynamic range, and is compatible with melting curve analysis.

Special stabilizers are included in the SuperMix to reduce nonspecific amplification products. Ultrapure, nonacetylated BSA is included because the glass capillaries in the LightCycler® have a high surface-to-volume ratio and the glass surface binds molecules such as *Taq* DNA polymerase. The addition of BSA blocks this surface binding.

A tube of 50-mM MgCl₂ is included in the SuperMix for further optimization of the Mg²⁺ concentration. Reagents are provided for 96 or 480 amplification reactions of 20 µl each.

Component

2.5X SYBR® Green One-Step SuperMix with BSA
50-mM Magnesium Chloride (MgCl₂)

96-Rxn Kit

770 µl
1 ml

480-Rxn Kit

3 × 1.3 ml
2 × 1 ml

Storage

Store components in the dark at -20°C.

Additional Products

Product

SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit with ROX

Amount

100 rxns
500 rxns

Catalog no.

11746-100
11746-500

PureLink™ Micro-to-Midi™ Total RNA Purification System

50 rxns

12183-018

TRIzol® Reagent

100 ml
200 ml

15596-026
15596-018

PureLink™ 96 Total RNA Purification Kit

384 rxns

12173-011

RNaseOUT™ Recombinant Ribonuclease Inhibitor

5000 units

10777-019

DNase I, Amplification Grade

100 units

18068-015

Custom Primers

to order, visit www.invitrogen.com

Part no. 11750.pps

Rev. date: 18 October 2010

Recommendations and Guidelines for Quantitative RT-PCR

RNA

- Starting material can range from 0.1 pg to 1 µg of purified total RNA. High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis and accurate mRNA quantification.
- RNA should be devoid of any RNase contamination and aseptic conditions should be maintained. **Optional:** An RNase inhibitor protein, such as RNaseOUT™ Recombinant Ribonuclease Inhibitor (Cat. no. 10777-019), may be added to the reaction after the SuperMix to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.
- To isolate total RNA, we recommend the PureLink™ Micro-to-Midi™ Total RNA Purification System (Cat. no. 12183-018), TRIzol® Reagent (Cat. nos. 15596-026 and 15596-018), or the PureLink™ 96 Total RNA Purification Kit for high-throughput applications (Cat. no. 12173-011). Isolation of mRNA from total RNA is typically not necessary, although incorporating this step may improve the yield of specific cDNAs.
- RNA may be treated with DNase I (Catalog no. 18068-015) to remove any contaminating genomic DNA.

Instrument Settings

This SuperMix is specifically formulated for use with the Roche LightCycler®. General instrument settings are provided in the protocol on page 3. Refer to your instrument manual for detailed operating instructions.

Melting Curve Analysis

Melting curve analysis should always be performed during real-time qPCR to identify the presence of primer dimers and analyze the specificity of the reaction.

Melting curve analysis can identify primer dimers by their lower melting temperature compared to that of the amplicon. The presence of primer dimers in samples containing template decreases PCR efficiency and obscures analysis and determination of cycle thresholds. The formation of primer dimers most often occurs in no-template controls, where the polymerase enzyme is essentially idle, and in this case the quantitative analysis of the template samples is not affected. Melting curve analysis of no-template controls can discriminate between primer dimers and spurious amplification due to contaminating nucleic acids in reagent components.

Reaction Setup and Conditions

- Keep all components and samples on ice.
- Efficient cDNA synthesis can be accomplished in a 2–10-minute incubation at 42–55°C. Optimal temperature varies for different primers and templates. A good general starting point is 50°C for 2 minutes. For problematic templates, or to increase the specificity of cDNA priming, increase the cDNA synthesis temperature to 55°C.
- During the 2-minute incubation at 95°C, SuperScript™ III RT is inactivated, the RNA/cDNA hybrid is denatured, and Platinum® Taq DNA polymerase is activated.
- The annealing temperature should be 0–10°C below the melting temperature of the primers used.
- The extension time of 10 seconds is appropriate for the short amplicons that are typically used in qPCR.

Primers

- Gene-specific primers are required. We do not recommend using oligo(dT) or random primers, which may generate nonspecific products in the one-step procedure and reduce the amount of product.
- Primer selection is one of the most important parameters when using a SYBR® Green detection system. To design primers, we strongly recommend using a primer design software program such as OligoPerfect™ Designer (on the Web at www.invitrogen.com/oligoperfect). In OligoPerfect™, you enter your target sequence and select PCR: *Detection* from the Application pulldown menu to access the appropriate design parameters for qPCR applications. Invitrogen's Vector NTI™ software can also be used to design primers. Primer design software will ensure that primers are specific for the target sequence and free of internal secondary structure, and avoid complementation at 3'-ends within each primer and with each other.
- Design primers that anneal to exons on both sides of an intron or within the exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and potential contaminating genomic DNA.
- For best results, the amplicon size should be limited to 80–200 bp.
- A final concentration of 500 nM per primer is effective for most reactions. Optimal results may require a primer titration.

Magnesium Concentration

The 2.5X SuperMix supplies a final magnesium concentration of 3 mM. This works well for most targets; however, the optimal magnesium concentration in qRT-PCR may vary between 3 and 6 mM.

If necessary, use the 50-mM magnesium chloride solution included in the SuperMix to increase the magnesium concentration. Use the following table to determine the amount of additional MgCl₂ necessary to achieve the specified concentration (in a 20-µl reaction with 8 µl of SuperMix):

<u>For a final MgCl₂ conc. of</u>	<u>Add 50-mM MgCl₂ (per 20-µl Rxn)</u>
4.0 mM	0.4 µl
5.0 mM	0.8 µl
6.0 mM	1.2 µl

Decrease the amount of water in the reaction accordingly.

Note: Increasing the magnesium concentration may also increase the level of nonspecific amplification products, such as primer dimers. To reduce the level of nonspecific products, you may need to reduce the concentration of primers in the reaction.

Roche LightCycler® Protocol

The protocol below summarizes the cycling conditions and reaction components for one-step qRT-PCR on the Roche LightCycler®. The SuperMix is provided at a 2.5X concentration to allow for 8 µl of SuperMix, 1 µl of each primer, and up to 10 µl of template in a 20-µl reaction volume.

1. Program the LightCycler® to perform cDNA synthesis immediately followed by PCR amplification, as specified below. Optimal temperatures and incubation times may vary for different target sequences (see **Reaction Setup and Conditions**, previous page).

PCR Cycling	Melting Curve Analysis
<i>Program choice:</i> Amplification	<i>Program choice:</i> Melting curve
<i>Analysis mode:</i> Quantification	<i>Analysis mode:</i> Melting curves
50°C for 2 minutes hold	95°C, 0 seconds
95°C for 2 minutes hold	55°C, 10 seconds
45 cycles of:	95°C, 10 seconds (slope set at 0.1°C/second, continuous acquisition)
95°C, 5 seconds	40°C, 0 seconds
55°C, 10 seconds (single acquire)	
72°C, 10 seconds	

2. Prepare a master mix on ice of all components except template. **Note:** Preparation of a master mix is **crucial** in quantitative applications to reduce pipetting errors.

Component	1 reaction	34 reactions
2.5X SYBR® Green One-Step SuperMix with BSA	8 µl	272 µl
Forward primer, 10 µM	1 µl	34 µl
Reverse primer, 10 µM	1 µl	34 µl
DEPC-treated water	x µl *	x µl *

* The amount of water depends on the amount of template you will add for a final reaction volume of 20 µl.

3. Add a single reaction volume of the master mix to each LightCycler® glass capillary tube.
4. Add 2–10 µl of sample RNA (0.1 pg to 1 µg total RNA) to each capillary tube, for a final reaction volume of 20 µl.
5. Gently mix and make sure that all components are at the bottom of the tube. Centrifuge briefly if needed.
6. Place reactions in the LightCycler® programmed as described above. Collect and analyze the results.

Quality Control

The product is tested functionally by quantitative real-time analysis using total HeLa RNA as template. Kinetic analysis must demonstrate a linear dose response with decreasing target concentration and detection of β -actin mRNA in 1 pg of total HeLa RNA.

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Troubleshooting Guide

Problem	Possible Cause	Probable Solution
No amplification product Relative fluorescent signal \leq background or no template control	Problem with instrument settings cDNA synthesis temperature too high, low priming efficiency RT or cDNA primer blocked by secondary structure RNA has been damaged or degraded RNase contamination	Run the sample on a gel to determine if PCR product was generated. If the appropriate bands are seen, check the instrument settings Lower incubation temperature. Raise incubation temperature. Redesign primer(s). Replace RNA if necessary. Maintain aseptic conditions.
Poor sensitivity, or product detected at higher than expected cycle number	Not enough starting template RNA RNA has been damaged or degraded RNase contamination RT inhibitors are present in RNA Inefficient cDNA synthesis Inefficient PCR amplification	Increase the concentration of template RNA; use 10 ng to 1 μ g of total RNA. Replace RNA if necessary. Store RNA at -70°C, limit the number of freeze-thaw cycles, and keep the template on ice before the reaction. Maintain aseptic conditions. Remove inhibitors in the RNA preparation by an additional 70% ethanol wash. Inhibitors of RT include SDS, EDTA, guanidium salts, formamide, sodium phosphate and spermidine (19, 20). Adjust cDNA synthesis temperature and/or primer design. Optimize PCR conditions: Adjust annealing temperature as necessary. Increase magnesium concentration. Redesign primers.
Product detected at lower-than-expected cycle number	Too much sample added to reactions	Decrease the concentration of template RNA.
Product detected at lower-than-expected cycle number, and/or positive signal from no-template controls	Template or PCR carry-over contamination	Isolate source of contamination and replace reagent(s). Use separate dedicated pipettors for reaction assembly and post-PCR analysis. Assemble reactions (except for target addition) in a DNA-free area. Use aerosol-resistant pipette tips or positive displacement pipettors.
Unexpected bands after electrophoresis	RNA contaminated with DNA Oligo(dT) or random primers used Low specificity in PCR Suboptimal primer design	Pre-treat RNA with DNase I. Use gene-specific primers. Optimize PCR conditions as described above. Redesign primers.

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