invitrogen™

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

Cat. no. 11750-100 **Size: 96 reactions**
Cat. no. 11750-500 **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 $MgCl_2$; 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 $MgCl_2$ is included in the SuperMix for further optimization of the Mg^{2+} concentration. Reagents are provided for 96 or 480 amplification reactions of 20 µl each.

Component	96-Rxn Kit	480-Rxn Kit
2.5X SYBR® Green One-Step SuperMix with BSA	770 µl	3 × 1.3 ml
50-mM Magnesium Chloride ($MgCl_2$)	1 ml	2 × 1 ml

Storage

Store components in the dark at -20ºC.

Additional Products

Product	Amount	Catalog no.
SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit with ROX	100 rxns	11746-100
	500 rxns	11746-500
PureLink™ Micro-to-Midi™ Total RNA Purification System	50 rxns	12183-018
TRIzol® Reagent	100 ml	15596-026
	200 ml	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 $MgCl_2$ necessary to achieve the specified concentration (in a 20-µl reaction with 8 µl of SuperMix):

For a final $MgCl_2$ conc. of	Add 50-mM $MgCl_2$ (per 20-µl Rxn)
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
Program choice: Amplification	*Program choice:* Melting curve
Analysis mode: Quantification	*Analysis mode:* Melting curves
50°C for 2 minutes hold	95ºC, 0 seconds
95°C for 2 minutes hold	55ºC, 10 seconds
45 cycles of:	95ºC, 10 seconds (slope set at 0.1ºC/second, continuous acquisition)
95ºC, 5 seconds	40ºC, 0 seconds
55ºC, 10 seconds (single acquire)	
72ºC, 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.

References

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Troubleshooting Guide

Problem	Possible Cause	Probable Solution
No amplification product Relative fluorescent signal ≤ background or no template control	Problem with instrument settings	Run the sample on a gel to determine if PCR product was generated. If the appropriate bands are seen, check the instrument settings
	cDNA synthesis temperature too high, low priming efficiency	Lower incubation temperature.
	RT or cDNA primer blocked by secondary structure	Raise incubation temperature. Redesign primer(s).
	RNA has been damaged or degraded	Replace RNA if necessary.
	RNase contamination	Maintain aseptic conditions.
Poor sensitivity, or product detected at higher than expected cycle number	Not enough starting template RNA	Increase the concentration of template RNA; use 10 ng to 1 µg of total RNA.
	RNA has been damaged or degraded	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.
	RNase contamination	Maintain aseptic conditions.
	RT inhibitors are present in RNA	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).
	Inefficient cDNA synthesis	Adjust cDNA synthesis temperature and/or primer design.
	Inefficient PCR amplification	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	Pre-treat RNA with DNase I.
	Oligo(dT) or random primers used	Use gene-specific primers.
	Low specificity in PCR	Optimize PCR conditions as described above.
	Suboptimal primer design	Redesign primers.