One-Step RT-PCR Kit

Cat. No.: SM300-0050 Size: 50 Reactions
Cat. No.: SM300-0010 Size: 10 Reactions
Store at -20°C

Description

The One-Step RT-PCR with HotStar *Taq* System is designed for the convenient, sensitive, and reproducible detection and analysis of RNA molecules by RT-PCR. Components for both cDNA synthesis and PCR are combined in a single tube, using gene-specific primers and target RNAs from either total RNA or mRNA. Reverse transcription automatically follows PCR cycling without additional steps. The kit consists of two major components: RT/ HotStar *Taq* Mix and 2X Reaction Mix. The RT/ HotStar *Taq* Mix contains a mixture of Reverse Transcriptase and HotStar *Taq* DNA Polymerase for optimal cDNA synthesis and PCR amplification. Reverse transcriptase is a modified version of Moloney Murine Leukemia Virus (M-MLV) RT, engineered to reduce RNase H activity and increase thermal stability. HotStar *Taq* DNA polymerase is *Taq* DNA polymerase complexed with a proprietary antibody that inhibits polymerase activity at ambient temperatures. The antibody is denatured and polymerase activity is restored during the denaturation step in PCR cycling at 94°C. This provides an automatic "hot start" in PCR, increasing sensitivity, specificity, and yield. The 2X Reaction Mix consists of a proprietary buffer system optimized for reverse transcription and PCR amplification, Mg^{2+} optimized for universal use, deoxyribonucleotide triphosphates, and stabilizers. This convenient 2X format allows further addition of template and primer at any desired concentration. One addition tube of $MgSO_4$ (50 mM) is included in the kit. Reagents are provided with the sufficient amplification reactions of 50 µl each.

Kit Content

Component	SM300-0050	SM300-0010
RT/ HotStar Taq Mix	50 µl	10 µl
2X Reaction Mix	1.25 ml	250 µl
50 mM Magnesium Sulfate	200 µl	40 µl

Important Parameters

RNA

- High quality intact RNA is essential for successful full-length cDNA synthesis.
- RNA should be devoid of any RNase contamination and aseptic conditions should be maintained.

Primers

- Gene specific primers (GSP) are recommended. Use of oligo(dT) or random primers are not recommended as they result in generation of non-specific products in the one-step procedure and the amount of RT-PCR product may be reduced.
- A final primer concentration of 0.2 µM for each primer is generally optimal. However, for best results, a primer titration using 0.15–0.5 µM is recommended.
- Design primers that anneal to sequence in exons on both sides of an intron or exon/exon boundary of the mRNA to allow differentiation between amplification of cDNA and potential contaminating genomic DNA.
- Primers should not be self-complementary or complementary to each other at the 3' ends.

Magnesium Concentration

- The 2X Reaction Mix includes magnesium at a final concentration of 3 mM. This works well for most targets; however, the optimal concentration may range from 3 to 6 mM. If necessary, use the separate tube of 50-mM magnesium sulfate to increase the magnesium concentration. Use the following table to determine the amount of $MgSO_4$ to add to achieve the specified concentration (in a 50-µl PCR with 25 µl of 2X Reaction Mix)

Volume of 50-mM $MgSO_4$ (per 50-µl Rxn)	Final $MgSO_4$ Conc.
1 µl	4.0 mM
2 µl	5.0 mM
3 µl	6.0 mM

Decrease the amount of water in the reaction accordingly

dNTPs

- 200 µM dNTP concentration is optimal for most RT-PCR reactions.

Recommendations and Tips

- Keep all components, reaction mixes, and samples on ice. After preparation of the samples, transfer them to a pre-heated thermal cycler (45-55°C, depending on the cDNA step temperature) and immediately start the RT-PCR amplification program.
- Efficient cDNA synthesis can be accomplished in a 15–30 minutes incubation at 45-55°C.
- The reverse transcriptase is inactivated, HotStar *Taq* DNA polymerase is reactivated and the RNA/cDNA hybrid is denatured during the 2 minutes incubation at 94°C.
- The annealing temperature should be 10°C below the melting temperature of the primers used.
- The extension time varies with the size of the amplicon (approximately 1 minutes per 1 kb of amplicon).
- For all targets up to 3 kb, 1 µl of RT/ HotStar *Taq* Mix is sufficient.

Quality Control

The product is tested functionally using 10 pg of total HeLa RNA as the template for amplification of a 353-bp segment of β-actin mRNA (40 cycles). A minimum of 25 ng of the RT-PCR product was obtained under these conditions.

Protocol

1. Program the thermal cycler so that cDNA synthesis is followed immediately with PCR amplification automatically.
   Note: The following cycling conditions were established using a DNA Thermal Cycler 9600 or 2400 (Perkin Elmer) and may have to be altered for other thermal cyclers. Efficient cDNA synthesis can be achieved in a 15-30 minutes incubation at 45-55°C. It is recommended that a 30-minute incubation at 50°C be used as a general starting point. The optimal temperature for reverse transcription will depend on primer and target sequences. Remember that cycling conditions may have to be further optimized for different sequences. Annealing and extension steps are separate (three-step cycling).
2. Add the following to the microcentrifuge tubes placed on ice.
   Reaction cocktails can be made when multiple reactions are being assembled.

Components	Volume/50 µl	Final Concentration
2X Reaction Mix	25 µl	1X
Template RNA	x µl	10 pg – 1 µg
Sense Primer (10 µM)	1 µl	0.2 µM
Anti-sense Primer (10 µM)	1 µl	0.2 µM
RT/ HotStar Taq Mix	1 µl	—
Autoclaved distilled water	to 50 µl	—

   Note: Absence of genomic DNA in RNA preparations can be verified by omitting the RT/ HotStar *Taq* Mix and substituting 2 units of HotStar Taq DNA polymerase in the reaction.

3. Gently mix and make sure that all the components are at the bottom of the amplification tube. Centrifuge briefly if needed. Depending on the thermal cycler used, overlay with silicone oil, if necessary.
4. Process in the thermal cycler for 35-40 cycles as follows:

cDNA synthesis	45-55°C for 15-30 minutes	
Pre-denaturation	94°C for 2 minutes	
PCR amplification		
Denature	94°C for 15 seconds	35-40 cycles
Anneal	55-60°C for 30 seconds	
Extend	68-72°C for 1 minute/kb	
Final extension (optional)	72°C for 5-10 minutes	

   For use in Perkin-Elmer Model 480 cycler, use 30 seconds denaturation instead of 15 seconds.
5. Analyze the amplification product.

Troubleshooting Guide

Problem	Possible cause	Possible solution
No amplification product	• No cDNA synthesis (temperature too high) • RNase contamination • Not enough starting template RNA	• For the cDNA synthesis step, incubate <55°C. • Increase the concentration of template RNA; use 100 ng to 1 µg of total RNA.
	• RNA has been damaged or degraded • RT inhibitors are present in RNA	• Replace RNA if necessary. • Remove inhibitors in the RNA preparation by an additional 70% ethanol wash. NOTE: Inhibitors of RT include SDS, EDTA, guanidium salts, formamide, sodium phosphate and spermidine.
	• Annealing temperature is too high	• Decrease temperature as necessary.
	• Extension time is too short	• Set extension time for at least 60 seconds per kb of target length.
Low specificity	• Reaction conditions not optimal	• Optimize magnesium concentration. • Optimize the primer. • Optimize the annealing temperature and extension time. • Increase temperature of RT reaction to 50-55°C.
	• Oligo(dT) or random primers used for first strand synthesis	• Use gene-specific primers.
Unexpected bands after electrophoresis	• RNA contamination with genomic DNA	• Pre-treat RNA with DNase I.