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SuperScript® III First-Strand Synthesis System for RT-PCR

Catalog. no.
18080-051

Size
50 reactions

Store at –30°C to –10°C

Doc. Part No 18080051.pps **Pub. No.** MAN0001346 **Rev.** 3.0

Description

The SuperScript® III First-Strand Synthesis System for RT-PCR is optimized to synthesize first-strand cDNA from purified poly(A)$^+$ or total RNA. RNA targets from 100 bp to >12 kb can be detected with this system. The amount of starting material can vary from 1 pg–5 µg of total RNA. 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. The enzyme is used to synthesize cDNA at a temperature range of 42–55°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 may be used to synthesize first-strand cDNA from a total RNA preparation.

cDNA synthesis is performed in the first step using either total RNA or poly(A)$^+$-selected RNA primed with oligo(dT), random primers, or a gene-specific primer. In the second step, PCR is performed in a separate tube using primers specific for the gene of interest. For the PCR reaction, we recommend one of the following DNA polymerases: Platinum® *Taq* DNA Polymerase provides automatic hot-start conditions for increased specificity up to 4 kb, Platinum® *Taq* DNA Polymerase High Fidelity provides increased yield and high fidelity for targets up to 15 kb, and Platinum® *Pfx* DNA Polymerase provides maximum fidelity for targets up to 12 kb.

Contents

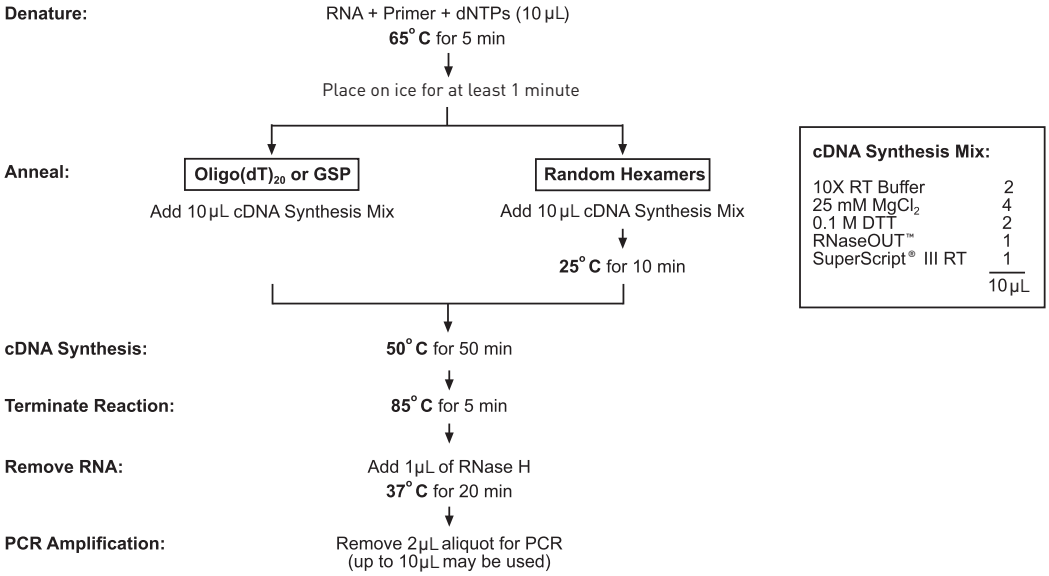
System Component	Amount
Oligo(dT)$_{20}$ (50 µM)	50 µL
Random hexamers (50 ng/µL)	250 µL
10X RT buffer*	1 mL
25 mM $MgCl_2$	500 µL
0.1 M DTT	250 µL
10 mM dNTP mix	250 µL
SuperScript® III RT (200 U/µL)	50 µL
RNaseOUT™ (40 U/µL)	100 µL
E. coli RNase H (2 U/µL)	50 µL
DEPC-treated water	1.2 mL
Total HeLa RNA (10 ng/µL)	20 µL
Sense Control Primer (10 µM)	25 µL
Antisense Control Primer (10 µM)	25 µL

*200 mM Tris-HCl (pH 8.4), 500 mM KCl

Related Products

	Amount	Catalog No.
Platinum® *Taq* DNA Polymerase	100 units	10966-018
	250 units	10966-026
	500 units	10966-034
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
	500 units	11304-029
Platinum® *Pfx* DNA Polymerase	100 units	11708-013
	250 units	11708-021
	500 units	11708-039
PCR$_x$ Enhancer System	250 rxns	11495-017
TRIzol® Reagent	100 mL	15596-026
	200 mL	15596-018
DNase I, Amplification Grade	100 units	18068-015
Custom Primers	to order, visit **www.lifetechnologies.com**	

Summary of Procedure



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Recommendations and Guidelines for First-Strand Synthesis

RNA

- High-quality, intact RNA is essential for full-length, high-quality cDNA synthesis. This kit is designed for use with 1 pg–5 µg of total RNA or 1 pg–500 ng of poly(A)$^+$ RNA. For >5 µg total RNA, increase reaction volumes and amount of SuperScript® III RT proportionally.
- RNaseOUT™ Recombinant RNase Inhibitor has been added to the system to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.
- To isolate total RNA, we recommend the Micro-to-Midi Total RNA Purification System (Cat. no. 12183-018), TRIzol® Reagent (Cat. nos. 15596-026/-018), or the Chomczynski and Sacchi method. Oligo (dT)-selection for poly(A)$^+$ RNA is typically not necessary, although it may improve the yield of specific cDNAs.
- Small amounts of genomic DNA in the RNA preparation may be amplified along with the target cDNA. If your application requires removal of all genomic DNA from your RNA preparation, we recommend using DNase I, Amplification Grade (Catalog no. 18068-015). DNase I, Amplification Grade, has been extensively purified to remove trace ribonuclease activities commonly associated with other "RNase-free" enzyme preparations, and does not require the addition of placental RNase inhibitor.

RNase H Digestion

The sensitivity of the PCR step can be increased (especially for long templates) by removing the RNA template from the cDNA:RNA hybrid molecule by digestion with RNase H after first-strand synthesis. Presence of RNase H during first-strand synthesis degrades the template mRNA, resulting in decreased full-length cDNA synthesis and decreased yields of first-strand cDNA. The SuperScript® III First-Strand Synthesis System introduces RNase H activity only when it is beneficial, and thus offers a unique procedural advantage over other methods.

First-Strand cDNA Synthesis

The following procedure is designed to convert 1 pg–5 µg of total RNA or 1 pg–500 ng of poly(A)$^+$ RNA into first-strand cDNA:

1. Mix and briefly centrifuge each component before use.
2. Combine the following in a 0.2- or 0.5-mL tube:

Component	Amount
up to 5 µg total RNA	*n* µL
Primer* *50 µM oligo(dT)$_{20}$, *or* 2 µM gene-specific primer (GSP), *or* 50 ng/µL random hexamers	1 µL
10 mM dNTP mix	1 µL
DEPC-treated water	to 10 µL

3. Incubate the tube at 65°C for 5 min, then place on ice for at least 1 min.
4. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

Component	1 Rxn	10 Rxns
10X RT buffer	2 µL	20 µL
25 mM $MgCl_2$	4 µL	40 µL
0.1 M DTT	2 µL	20 µL
RNaseOUT™ (40 U/µL)	1 µL	10 µL
SuperScript® III RT (200 U/µL)	1 µL	10 µL

5. Add 10 µL of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.
   Oligo(dT)$_{20}$ or GSP primed: 50 min at 50°C
   Random hexamer primed: 10 min at 25°C, followed by 50 min at 50°C
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µL of RNase H to each tube and incubate the tubes for 20 min at 37°C.
8. cDNA synthesis reaction can be stored at –30°C to –10°C or used for PCR immediately.

Primers

The first-strand cDNA synthesis reaction can be primed using random hexamers, oligo(dT), or gene-specific primers (GSPs):

- Random hexamers are the most nonspecific priming method, and are typically used when the mRNA is difficult to copy in its entirety. With this method, all RNAs in a population are templates for first-strand cDNA synthesis, and PCR primers confer specificity during PCR. To maximize the size of cDNA, you should determine the ratio of random hexamers to RNA empirically for each RNA preparation.

  Note: For most RT-PCR applications, 50 ng of random hexamers per 5 µg of total RNA is adequate. Increasing hexamers to 250 ng per 5 µg of RNA may increase yield of small PCR products (<500 bp), but may decrease the yield of longer PCR products and full-length transcripts.
- Oligo(dT), a more specific priming method, is used to hybridize to 3´ poly(A) tails, which are found in the vast majority of eukaryotic mRNAs. Since poly(A)$^+$ RNA constitutes approximately 1% to 2% of total RNA, the amount and complexity of cDNA is considerably less than with random hexamers. We recommend using oligo(dT)$_{20}$ (provided in the kit).

  Note: Oligo(dT) is recommended over random hexamers or GSPs when performing RT-PCR with new mRNA targets. Oligo(dT) produces an RT-PCR product more consistently than random hexamers or GSPs.
- The most specific priming method uses a gene-specific primer for the sequence of interest. First-strand synthesis can be primed with the PCR primer that hybridizes nearest to the 3´ terminus of the mRNA. Note that some GSPs fail to prime cDNA synthesis even though they work in PCR on DNA templates. If gene-specific priming fails in RT-PCR, repeat first-strand synthesis using oligo(dT) as the primer.

Amplification of Target cDNA

The first-strand cDNA obtained in the synthesis reaction may be amplified directly using PCR. We recommend using 10% of the first-strand reaction (2 µL) for PCR. However, for some targets, increasing the amount of first-strand reaction up to 10 µL in PCR may result in increased product yield.

We recommend the following DNA polymerases (for ordering information, see page 1):

- **Platinum® *Taq* DNA Polymerase** provides automatic hot-start conditions for increased specificity and sensitivity. It is recommended for targets up to 4 kb.
- **Platinum® *Taq* DNA Polymerase High Fidelity** provides increased fidelity and higher yields for targets up to 15 kb.
- **Platinum® *Pfx* DNA Polymerase** possesses a proofreading 3´ to 5´ exonuclease activity and provides maximum fidelity for PCR. It is recommended for targets up to 12 kb.

Consult the product documentation provided with each DNA polymerase for recommended protocols and optimization guidelines. Documentation is also available at **www.lifetechnologies.com**.

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Control Reactions

The control RNA provided with this system consists of total HeLa RNA (10 ng/µL). The sense and antisense control primers provided with this kit are designed from the human β-actin gene and produce a 353-bp RT-PCR product.

Sense primer: 5'-GCTCG TCGTC GACAA CGGCT C-3'

Antisense primer: 5'-CAAAC ATGAT CTGGG TCATC TTCTC-3'

Use the following protocol for both plus and minus RT control reactions:

1. Dilute the total HeLa RNA to 100pg/µL with DEPC-treated water.
2. Prepare the RNA/primer mixtures in sterile 0.2- or 0.5-mL tubes as follows:

Component	+ RT Control	– RT Control
Diluted total HeLa RNA (100 pg/µL)	1 µL	1 µL
Oligo(dT)$_{20}$	1 µL	1 µL
10 mM dNTP mix	1 µL	1 µL
DEPC-treated water	7 µL	7 µL

3. Incubate the samples at 65°C for 5 min, then place them on ice for at least 1 min. Collect the contents by brief centrifugation and add the following:

Component	+ RT Control	– RT Control
10X RT buffer	2 µL	2 µL
25 mM $MgCl_2$	4 µL	4 µL
0.1 M DTT	2 µL	2 µL
RNaseOUT™ (40 U/µL)	1 µL	1 µL
SuperScript® III RT (200 U/µL)	1 µL	—
DEPC-treated water	—	1 µL

4. Mix the tubes gently and collect the reactions by brief centrifugation.
5. Incubate the samples at 50°C for 50 min.
6. Terminate the reactions at 85°C for 5 min. Chill on ice.
7. Collect the reactions by brief centrifugation. Add 1 µL of RNase H to each tube and incubate for 20 min at 37°C.
8. Prepare a PCR mixture for each control reaction. For each control reaction, add the following to a 0.2-mL tube sitting on ice:

Component	Volume
DEPC-treated water	38.1 µL
10X PCR buffer minus Mg^{++}	5 µL
50 mM $MgCl_2$	1.5 µL
10 mM dNTP mix	1 µL
Control sense primer (10 µM)	1 µL
Control antisense primer (10 µM)	1 µL
cDNA from control RNA	2 µL
Taq DNA polymerase (5 units/µL)	0.4 µL
Final volume	50 µL

9. Mix the contents of the tube. Centrifuge briefly to collect the reaction components.
10. Place reaction mixture in preheated (94°C) thermal cycler. Perform an initial denaturation step: 94°C for 2 min.
11. Perform 40 cycles of PCR:

Denature	94°C for 15 sec
Anneal	55°C for 30 sec
Extend	68–72°C for 1 min

Note: For slow-ramping thermal cyclers, follow manufacturer's directions.

12. Upon completion, maintain reactions at 4°C.
13. Analyze 10 µL of each reaction, using agarose gel electrophoresis and ethidium bromide staining. For the + RT Control, a 353-bp band, corresponding to at least 25 ng of product, should be visible. For the – RT Control, the same band should be ≤50% in intensity when compared to the + RT Control.

First Strand cDNA Synthesis of Transcripts with High GC Content

High-GC content mRNAs often contain stable intrinsic secondary structures that can inhibit reverse transcriptase and/or primer annealing. Problems with RT-PCR due to this secondary structure often can be overcome by increasing the volume and temperature of the RT reaction.

Note: For templates that require cDNA synthesis temperatures above 55°C, we recommend the ThermoScript™ RT-PCR System (Catalog no. 11146-024). ThermoScript™ RT supports cDNA synthesis up to 70°C.

This protocol is suitable for gene-specific or oligo(dT) primers, but not random hexamers.

1. Mix and briefly centrifuge each component before use.
2. Prepare the RNA/primer mixture in a sterile 0.5-mL tube as follows:

Component	Sample	Control RNA
1–5 µg total RNA	*n* µL	—
Control total HeLa RNA (10 ng/µL)	—	1 µL
Oligo(dT)$_{20}$ (50 µM) *or* 2 µM GSP	1 µL	1 µL
10 mM dNTP mix	2.5 µL	2.5 µL
DEPC-treated water	to 25 µL	to 25 µL

3. Incubate each sample at 65°C for 5 min and immediately transfer to 55°C.
4. Prepare the cDNA Synthesis Mix, adding each component in the indicated order.

Component	1 Reaction	10 Reactions
DEPC-treated water	3 µL	30 µL
10X RT buffer	5 µL	50 µL
25 mM $MgCl_2$	10 µL	100 µL
0.1 M DTT	5 µL	50 µL
RNaseOUT™ Recombinant RNase Inhibitor	1 µL	10 µL
SuperScript® III RT	1 µL	10 µL

Note: For a minus RT control reaction, substitute 1 µL of DEPC-treated water for 1 µL of SuperScript® III RT, and assemble reaction as described above.

5. Prewarm the cDNA Synthesis Mix to 55°C.
6. To each sample incubating at 55°C, add 25 µL of prewarmed cDNA Synthesis Mix. Mix gently, and incubate at 55°C for 50 min.
7. Terminate the reactions at 85°C for 5 min. Chill on ice.
8. Collect the reactions by brief centrifugation. Add 1 µL of RNase H to each tube and incubate for 20 min at 37°C before proceeding to PCR.

   Note: Frequently, problems associated with RT-PCR of GC-rich cDNA are related to PCR as well as first-strand synthesis. We recommend using the PCR$_x$ Enhancer System (Catalog no. 11495-017) to facilitate amplification of GC-rich sequences.

Troubleshooting Guide

Problem	Possible Cause	Probable Solution
No bands after analysis of amplified products	Procedural error in first-strand cDNA synthesis	Use the total HeLa RNA provided as a control to verify the efficiency of the first-strand reaction (see page 3).
	RNase contamination	Add control RNA to sample to determine if RNase is present in the first-strand reaction. Maintain aseptic conditions to prevent RNase contamination. Use RNaseOUT™ Recombinant RNase Inhibitor in the first-strand reaction.
	Polysaccharide coprecipitation of RNA	Precipitate RNA with lithium chloride to remove polysaccharides, as described in Sambrook *et al.*
	Target mRNA contains strong transcriptional pauses	Use random hexamers instead of oligo(dT) in the first-strand reaction. Maintain an elevated temperature after the annealing step, as described in the protocol for cDNA synthesis from high-GC content transcripts, page 3. Increase the temperature of first-strand reaction (up to 55°C). Use PCR primers closer to the 3´ terminus of the target cDNA.
	Too little first-strand product was used in PCR	Use up to 10 µL of the first-strand reaction.
	GSP was used for first-strand synthesis	Try another GSP or switch to oligo(dT). Make sure the GSP is the antisense sequence.
	Inhibitors of RT present	Remove inhibitors by ethanol precipitation of mRNA preparation before the first-strand reaction. Include a 70% (v/v) ethanol wash of the mRNA pellet. **Note:** Inhibitors of RT include sodium dodecyl sulfate (SDS), EDTA, guanidinium salts, formamide, sodium pyrophosphate, and spermidine.
Unexpected bands after electrophoretic analysis	Contamination by genomic DNA	Pretreat RNA with DNase I, Amplification Grade (Cat. no. 18068-015), as described in the DNase I documentation. Design primers that anneal to sequence in exons on both sides of an intron or at the exon/exon boundary of the mRNA to differentiate between amplified cDNA and potential contaminating genomic DNA. To test if products were derived from DNA, perform the minus RT control.
	Nonspecific annealing of primers	Vary the annealing conditions. Use Platinum® *Taq* DNA Polymerase for automatic hot-start PCR. Optimize magnesium concentration for each template and primer combination.
	Primers formed dimers	Design primers without complementary sequences at the 3´ ends.

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