Second Strand cDNA Synthesis Kit

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Sheets (SDSs) are available from **thermofisher.com/support.**

Product description

Invitrogen™ Second Strand cDNA Synthesis Kit is designed to produce double-stranded cDNA using first strand reaction without the need for intermediate organic extraction or ethanol precipitation steps. This convenient one-tube format speeds up the synthesis procedure and maximizes cDNA recovery. The kit contains premixed components to reduce the number of pipetting steps necessary to complete the procedure.

Contents and storage

Cat. No.	Contents	Amount	Storage
A48570 50 rxns	Second Strand Enzyme Mix	1 mL	-25 °C to -15 °C
	5X Second Strand Reaction Mix	250 µL	
	0.5 M EDTA, pH 8.0	1 mL	
	RNase I, 10 U/µL	500 µL	
	Water, nuclease-free	3 x 1.25 mL	
A48571 200 rxns	Second Strand Enzyme Mix	4 x 1 mL	-25 °C to -15 °C
	5X Second Strand Reaction Mix	4 x 250 µL	
	0.5 M EDTA, pH 8.0	4 x 1 mL	
	RNase I, 10 U/µL	4 x 500 µL	
	Water, nuclease-free	12 x 1.25 mL	

Second Strand cDNA Synthesis Reaction

Second strand cDNA is generated using the first strand cDNA as a template. The Second Strand cDNA Synthesis Kit uses nick translational replacement of the mRNA to synthesize the second strand cDNA. First described by Okayama and Berg (1), and later popularized by Gubler and Hoffman (2), second strand cDNA synthesis is catalyzed by *E. coli* DNA polymerase I in combination with *E. coli* RNase H and *E. coli* DNA ligase. *E.coli* RNase H inserts nicks into the RNA, providing 3' OH-primers for DNA polymerase I. The 5'-3' exonuclease activity of *E. coli* DNA polymerase I removes the RNA strand in the direction of synthesis, while its polymerase activity replaces the RNA with deoxyribonucleotides. *E. coli* DNA ligase links the gaps to complete the ds cDNA strand.
The second strand reaction is performed at 16 °C to prevent spurious synthesis by DNA polymerase I due to its tendency to strand-displace (rather than nick translate) at higher temperatures.

Important Notes

Avoiding ribonuclease contamination

RNA purity and integrity is essential for synthesis of full-length cDNA. RNA can be degraded by RNase A, which is a highly stable contaminant found in any laboratory environment. All kit components have been rigorously tested to ensure that they are RNase-free.
To prevent contamination, both the laboratory environment and all prepared solutions must be free of RNases.
General recommendations to avoid RNase contamination:

- Use certified nuclease-free labware or DEPC-treat all tubes and pipette tips to be used in cDNA synthesis.
- Wear gloves when handling RNA and all reagents, as skin is a common source of RNases. Change gloves frequently.
- Use RNase-free reagents, including high quality water (e.g., Nuclease-Free Water (not DEPC-Treated) #AM9939).
- Ensure that the kit components are tightly sealed when not in use. Keep all tubes tightly closed during the reverse transcription reaction.

For First Strand Synthesis Reaction Protocol using Invitrogen™ SuperScript™ IV (#18090050), see page 3.

Protocol

I. Second Strand cDNA Synthesis

Thaw reaction components, mix, briefly centrifuge and place on ice.

1. Terminate the first strand reaction by heating at 85 °C for 5 minutes.
2. For one 100 µL reaction, pipette the following components directly into the first strand reaction tube on ice in the indicated order:

Component	Amount
First Strand cDNA Synthesis Reaction Mixture	20 µL
Water, nuclease-free	55 µL
5X Second Strand Reaction Mix	20 µL
Second Strand Enzyme Mix	5 µL
Total volume:	**100 µL**

3. Mix gently and centrifuge briefly.
4. Incubate at 16 °C for 60 min.
5. Stop the reaction by adding 6 µL 0.5 M EDTA, pH 8.0 and mixing gently.
6. Keep the reaction on ice (4 °C) until you are ready to continue with residual RNA removal or ds cDNA purification procedures.

II. Removal of RNA

If total RNA was used as a starting material, we recommend the following steps to remove residual RNA from the ds cDNA preparation:

1. Add 10 µL (100 U) RNase I to the second strand synthesis reaction tube.
2. Incubate for 5 minutes at room temperature.
3. Proceed with cDNA purification or store the reaction mixture at -20 °C.

III. Purification of ds cDNA

Purify blunt-end double-stranded cDNA using the PureLink™ PCR Micro Kit #K310010 for use in downstream applications immediately or freeze at -20 °C.

IV. Analysis of cDNA products

The quantity and size distribution of the synthesized ds cDNA can be estimated spectrophotometrically by measuring the absorbance at 260 nm (e.g. using NanoDrop™ ND-1000) and by gel analysis.

V. Downstream applications

Cloning of ds cDNA

Following purification, the blunt-end double-stranded cDNA can be used in the following cloning applications:

- direct ligation.
- ligation with any other blunt-end vector.
- ligation with specially designed adaptors, enabling cloning into sticky-end vectors. The choice of the adaptors depends on the vector used.

Restriction Digestion of ds cDNA

Following purification, blunt-end ds cDNA can be used in restriction enzyme digestion applications.

Troubleshooting

Problem	Cause and Solution
Low yield of second strand cDNA products	**Incomplete RT inactivation.** Always perform the RT inactivation step after first strand cDNA synthesis. **Improper preparation of the second strand reaction mixture.** Dilute the first strand reaction as outlined in the Second Strand cDNA Synthesis. Failure to properly dilute the first strand reaction changes the pH of the second strand reaction and influences the activity of the 3'-5' and 5'-3' exonuclease activities of DNA polymerase I. **Incorrect reaction temperature.** The second strand reaction must be incubated at 16 °C to prevent spurious synthesis by DNA polymerase I. At higher temperatures DNA polymerase I tends to strand-displace rather than nick translate.

SuperScript™ IV First-Strand cDNA Synthesis Reaction

The example procedure below shows appropriate volumes for a single **20-µL** reverse transcription reaction. For multiple reactions, prepare a master mix of components common to all reactions to minimize pipetting error, then dispense appropriate volumes into each reaction tube prior to adding annealed template RNA and primers.

I. Anneal primer to template RNA

1. Combine the following components in a reaction tube.
   Note: Consider the volumes for all components listed in steps I and II to determine the correct amount of water required to reach your final reaction volume.

Component	Amount
50 µM Oligo $d(T)_{20}$ primer, 50 µM random hexamers, or 2 µM gene-specific reverse primer	1 µL
10 mM dNTP mix (10 mM each)	1 µL
Template RNA	up to 11 µL
DEPC-treated or nuclease-free water	to 13 µL

2. Mix and briefly centrifuge the components.
3. Heat the RNA-primer mix at 65 °C for 5 minutes, and then incubate on ice for at least 1 minute.

II. Prepare RT reaction mix

1. Vortex and briefly centrifuge the 5X SSIV Buffer.
2. Combine the following components in a reaction tube.

Component	Amount
5X SSIV Buffer	4 µL
100 mM DTT	1 µL
RNaseOUT™ Recombinant RNase Inhibitor	1 µL
SuperScript™ IV Reverse Transcriptase (200 U/µL)	1 µL

3. Cap the tube, mix, and then briefly centrifuge the contents.

III. Combine annealed RNA and RT reaction mix

Add RT reaction mix to the annealed RNA.

IV. Incubate reactions

1. If using random hexamer, incubate the combined reaction mixture at 23 °C for 10 minutes, and then proceed to step 2.
   If using oligo $d(T)_{20}$ or gene-specific primer, directly proceed to step 2.
2. Incubate the combined reaction mixture at 50–55 °C for 10 minutes.
3. Inactivate the reaction by incubating it at 80 °C for 10 minutes.

References

1. Okayama, H., Berg, P. High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* 2, 161-170, 1982.
2. Gubler, K., Hoffmann, B. J. A simple and very efficient method for generating cDNA libraries. *Gene* 25, 263-269, 1983.
3. Fleige, S., Pfaffl, M.W., RNA integrity and the effect on the real-time qRT-PCR performance, Mol. Aspects Med., 27, 126-139, 2006.
4. Nolan, T., et al., Quantification of mRNA using real-time RT-PCR, Nat. Protoc., 1, 1559-1582, 2006.
5. Ausubel, F.M. et al. Current Protocols in Molecular Biology. *John Wiley and Sons*, Section 5.5, 1

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