# SuperScript® Double-Stranded cDNA Synthesis Kit

**Catalog no. 11917-010** **Size: 10 reactions**
**Catalog no. 11917-020** **Size: 50 reactions**

**Store at –20°C**

## Description

The SuperScript® Double-Stranded cDNA Synthesis Kit contains all of the reagents—except an oligo(dT) containing primer—necessary to make double-stranded cDNA from total RNA or poly $A^+$ selected RNA (mRNA).

It is important to use high-quality RNA for your cDNA preparation. High-quality RNA can be obtained by isolating total RNA with TRIzol® Reagent.

## Components

See the table below for the quantities of reagents in each kit:

| | Amounts | |
|---|---|---|
| **Component** | **10 rxn kit** | **50 rxn kit** |
| 5X First-Strand Reaction Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] | 1 mL | 1 mL |
| 0.1 M DTT | 100 µL | 100 µL |
| 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP) | 100 µL | 200 µL |
| SuperScript® II RT (200 U/µL) | 25 µL | 100 µL |
| 5X Second-Strand Reaction Buffer [100 mM Tris-HCl (pH 6.9), 450 mM KCl, 23 mM $MgCl_2$, 0.75 mM β-$NAD^+$, 50 mM $(NH_4)_2SO_4$] | 500 µL | 1500 µL |
| *E. coli* DNA Ligase (10 U/µL) | 15 µL | 50 µL |
| *E. coli* DNA Polymerase I (10 U/µL) | 50 µL | 200 µL |
| *E. coli* RNase H (2 U/µL) | 20 µL | 50 µL |
| T4 DNA Polymerase (5 U/µL) | 25 µL | 100 µL |
| DEPC-treated water | 2.5 mL | 3 × 2 mL |

## Intended Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

## Product Qualification

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to **www.invitrogen.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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## First-Strand Synthesis

The 20-µL reaction described in this protocol is designed to convert 25–50 µg of total RNA or 0.2–5 µg of mRNA into first-strand cDNA. The amount of SuperScript® II RT added to the reaction will depend on the amount of starting RNA. For total RNA, we recommend 200 units of SuperScript® II RT for ≤25 µg of total RNA and 400 units for 50 µg of total RNA. For mRNA, we recommend 200 units of SuperScript® II RT per µg of mRNA.

1. For priming with oligo dT-containing primer, add primer, RNA and DEPC-treated water according to the table below to a RNase-free 1.5-mL microcentrifuge tube:

| | µg of mRNA | | | | | µg of Total RNA | |
|---|---|---|---|---|---|---|---|
| | ≤1 | 2 | 3 | 4 | 5 | ≤25 | 50 |
| Primer (100 pmol/ul) | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| RNA in DEPC-treated water (ul) | 10 | 9 | 8 | 7 | 6 | 10 | 9 |
| Total volume (ul) | 11 | 10 | 9 | 8 | 7 | 11 | 10 |

2. Heat the mixture to 70°C for 10 min, and quick-chill on ice. Collect the contents of the tube by brief centrifugation, and add the following.

| Component* | Volume (µL) |
|---|---|
| 5X First-Strand Reaction Buffer | 4 |
| 0.1 M DTT | 2 |
| 10 mM dNTP mix | 1 |

* Optional: To analyze the first-strand yield, also add 1 µL [α-$^{32}$P]dCTP (1 µCi/µL)

The total volume should now correspond to the following table:

| | µg of mRNA | | | | | µg of Total RNA | |
|---|---|---|---|---|---|---|---|
| | ≤1 | 2 | 3 | 4 | 5 | ≤25 | 50 |
| Total volume (µL) | 19 | 18 | 17 | 16 | 15 | 19 | 18 |

3. Vortex gently and collect the reaction by brief centrifugation. Place the tube at 45°C for 2 min to equilibrate the temperature.
4. Add SuperScript® II RT according to the following table:

| | µg of mRNA | | | | | µg of Total RNA | |
|---|---|---|---|---|---|---|---|
| | ≤1 | 2 | 3 | 4 | 5 | ≤25 | 50 |
| SuperScript® II RT (µL) | 1 | 2 | 3 | 4 | 5 | 1 | 2 |

5. Mix gently, and incubate at 45°C for 1 h. The total volume should now be 20 µL.
6. Place the tube on ice to terminate the reaction.
7. If [α-$^{32}$P]dCTP was added, remove 2 µL from the reaction, and add it to a microcentrifuge tube containing 43 µL of 20 mM EDTA (pH 7.5) and 5 µL (1 µg/µL) of yeast tRNA. This mixture will be used in calculating first-strand yield. Take the remaining 18 µL of the first-strand reaction, and continue immediately with the first two steps of the second-strand reaction as described in **Second-Strand Synthesis**. If [α-$^{32}$P]dCTP was not added, proceed directly to **Second-Strand Synthesis**.
8. While the second-strand reaction is incubating, spot duplicate 10-µL aliquots from the diluted sample from step 7 above onto glass fiber filters. Dry one of the filters under a heat lamp or at room temperature. This filter will be used to determine the specific activity of the dCTP reaction.
9. Wash the other filter three times in sequence, for 5 min each time, in a beaker containing 50 mL of fresh, ice-cold 10% (w/v) TCA containing 1% (w/v) sodium pyrophosphate.
10. Wash the filter with 50 mL of 95% ethanol at room temperature for 2 min. Dry the filter under a heat lamp or at room temperature. This filter will be used to determine the yield of first-strand cDNA.
11. Count both filters in standard scintillant to determine the amount of $^{32}$P in the reaction, as well as the amount of $^{32}$P that was incorporated. See the next page for formulas to convert the data into yield of first-strand cDNA.
12. To analyze the cDNA product by gel analysis, precipitate the remaining 30 µL of the sample from step 7 of this protocol by adding 15 µL of 7.5 M $NH_4OAc$, followed by 90 µL of ice-cold absolute ethanol. Vortex the mixture thoroughly, and immediately centrifuge at room temperature for 20 min at 14,000 × *g*.
13. Remove the supernatant carefully, and overlay the pellet with 0.5 mL of ice-cold 70% ethanol. Centrifuge for 2 min at 14,000 × *g*, and remove the supernatant. Caution: If the first-strand cDNA was labeled, the supernatant(s) will be radioactive. Dispose of this material properly.
14. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol, and proceed to **Analysis of the cDNA Products**.

## Second-Strand Synthesis

This protocol is suitable for synthesizing second-strand cDNA from the 20-µL first-strand reaction.

1. On ice, add the following reagents in the order shown to the first-strand reaction tube:

| Component | Volume (µL) |
|---|---|
| DEPC-treated water | 91 |
| 5X Second-Strand Reaction Buffer | 30 |
| 10 mM dNTP mix | 3 |
| *E. coli* DNA Ligase (10 U/µL) | 1 |
| *E. coli* DNA Polymerase I (10 U/µL) | 4 |
| *E. coli* RNase H (2 U/µL) | 1 |
| Final volume | 150 |

2. Vortex gently to mix, and incubate for 2 h at 16°C. Do not allow the temperature to rise above 16°C.
3. Add 2 µL (10 units) of T4 DNA Polymerase, and continue to incubate at 16°C for 5 min.
4. Place the tube on ice, and add 10 µL of 0.5 M EDTA.
5. Add 160 µL of phenol:chloroform:isoamyl alcohol (25:24:1), vortex thoroughly, and centrifuge at room temperature for 5 min at 14,000 × *g* to separate the phases. Carefully remove 140 µL of the upper, aqueous layer, and transfer it to a fresh 1.5-mL microcentrifuge tube.
6. Add 70 µL of 7.5 M $NH_4OAc$, followed by 0.5 mL of ice-cold absolute ethanol. Vortex the mixture thoroughly, and immediately centrifuge at room temperature for 20 min at 14,000 × *g*.
7. Remove the supernatant carefully and discard. Overlay the pellet with 0.5 mL of ice-cold 70% ethanol. Centrifuge for 2 min at 14,000 × *g*, remove the supernatant and discard. **Caution: If either first or second-strand cDNA was labeled, the supernatant(s) will be radioactive. Dispose of this material properly.**
8. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol, and dissolve the pellet in a small volume of DEPC-treated water (3 µL per 25 µg of starting total RNA or 1 µg of starting mRNA).

## Analysis of the cDNA Products

The overall yield of the first-strand reaction is calculated from the amount of acid-precipitable radioactivity determined as described in **First-Strand Synthesis**. To perform the calculation, you must first determine the specific activity (SA) of the radioisotope in the reaction. The specific activity is defined as the counts per minute (cpm) of an aliquot of the reaction divided by the quantity (in pmol) of the nucleotide in the aliquot. For [α-$^{32}$P]dCTP, the specific activity is given by the relationship:

$$\text{SA (cpm/pmol dCTP)} = \frac{\text{cpm/10 µL}}{\text{200 pmol dCTP/10 µL}}$$

The amount of dCTP contributed by the radiolabeled material is insignificant relative to the unlabeled nucleotide and is ignored in equation 1.

Once the specific activity is known, the amount of cDNA in the first-strand reaction can be calculated from the amount of acid-precipitable radioactivity determined from the washed filter:

$$\text{Amount of cDNA (µg)} = \frac{\text{(cpm)} \times \text{(50 µL/10 µL)} \times \text{(20 µL/2 µL)} \times \text{(4 pmol dNTP/pmol dCTP)}}{\text{(cpm/pmol dCTP)} \times \text{(3,030 pmol dNTP/µg cDNA)}}$$

The correction in the numerator takes into account that, on the average, four nucleotides will be incorporated into the cDNA for every dCTP scored by this assay. The factor in the denominator is the amount of nucleotide that corresponds to 1 µg of single-stranded DNA.

**Example:** The unwashed filter gave 40,000 cpm when it was counted. The specific activity of the dCTP is given by equation 1:

$$\text{SA (cpm/pmol dCTP)} = \frac{\text{40,000 cpm/10 µL}}{\text{200 pmol dCTP/10 µL}} = 200$$

If 2 µg of starting mRNA was used and the washed filter gave 1,800 cpm, then the amount of cDNA is calculated using equation 2:

$$\text{Amount of cDNA (µg)} = \frac{\text{(1800 cpm)} \times \text{(50 µL/10 µL)} \times \text{(20 µL/2 µL)} \times \text{(4 pmol dNTP/pmol dCTP)}}{\text{(200 cpm/pmol dCTP)} \times \text{(3,030 pmol dNTP/µg cDNA)}}$$

$$= \quad 0.6 \text{ µg first-strand cDNA}$$

This amount of first-strand cDNA would represent a 30% yield relative to the 2 µg of mRNA starting material.

If 2 µg of starting mRNA was used and the washed filter gave 1,800 cpm, then the amount of cDNA is calculated using equation 2:

$$\text{Amount of cDNA (µg)} = \frac{(1800\text{cpm}) \times (50\ \mu\text{L}/10\ \mu\text{L}) \times (20\ \mu\text{L}/2\ \mu\text{L}) \times (4\ \text{pmol dNTP/pmol dCTP})}{(200\ \text{cpm/pmol dCTP}) \times (3{,}030\ \text{pmol dNTP/µg cDNA})} = 0.6\ \mu\text{g first strand cDNA}$$

This amount of first strand cDNA would represent a 30% yield relative to the 2 µg of mRNA starting material.

## Gel Analysis

The first strand cDNA, if labeled with $^{32}P$, can be analyzed by alkaline agarose gel electrophoresis to estimate the size range of products synthesized. Analysis of cDNA products by alkaline gel electrophoresis should reveal a size distribution of 0.5 to >7 kb. A lower size distribution suggests degraded RNA or RNase contamination.

## Troubleshooting

| Problem | Possible cause | Possible Solution |
|---|---|---|
| Low first strand cDNA yield | Temperature too high | For the cDNA synthesis incubate at 45°C. |
| | Incorrect reaction conditions used | Verify all reaction components were included in the reaction. Use reagents provided. |
| | Concentration of template RNA in reaction is too low | Increase the concentration of template RNA; use at least 25 µg of total RNA or 1 µg mRNA. |
| | Poor quality RNA | Isolate RNA using the TRIzol® Reagent or a guanidine isothiocyanate based homogenization procedure. |
| | RNase contamination | Maintain aseptic conditions; add RNaseOUT™ (RNase inhibitor). |
| | RNA has been damaged or degraded | Replace RNA. |
| | RT inhibitors are present in RNA | Remove inhibitors in the RNA preparation by an additional 70% ethanol wash after ethanol precipation.<br>Note: Inhibitors of RT include SDS, EDTA, guanidinium chloride, formamide, sodium phosphate and spermidine (1). |
| Low second strand yield | Incorrect reaction conditions used | Verify all reaction components were included in the reaction. Use reagents provided. |
| | Incorrect temperature | For second strand synthesis incubate at 16°C. |

## Reference

1. Gerard, G.F (1994). *Focus® 16*, 102.

## Purchaser Notification



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