Feb 24, 2026

High-Efficiency Double-Stranded cDNA Synthesis from dsRNA Templates

DOI
https://dx.doi.org/10.17504/protocols.io.5qpvobdybl4o/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.5qpvobdybl4o/v1

External link: http://dx.doi.org/10.17504/protocols.io.kxygxp64zl8j/v1

Protocol Citation: Abdonaser Poursalavati 2026. High-Efficiency Double-Stranded cDNA Synthesis from dsRNA Templates. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvobdybl4o/v1

Manuscript citation:
Pending publication of the associated manuscript, please cite this protocol using:
dx.doi.org/10.17504/protocols.io.kxygxp64zl8j/v1

Protocol status: Working
We use this protocol and it's working

Created: June 14, 2022

Last Modified: February 24, 2026

Protocol Integer ID: 64588

Keywords: dsRNA, ds-cDNA, cDNA synthesis, Viromics, RNA virome, SuperScript IV, Next-Generation Sequencing (NGS), Library Preparation, Soil Virome, SS-VIME, Custom Protocol, Environmental Metagenomics, Nucleic Acid Extraction, stranded cdna synthesis from dsrna templates high, stranded cdna synthesis from dsrna template, purified dsrna fraction, dsrna template, input dsrna, dsrna templates high, active rna virome, characterization of the active rna virome, using superscript iv reverse transcriptase, superscript iv reverse transcriptase, stranded cdna synthesis, rna, generation sequencing, sequencing library, microbiome extraction protocol toward comprehensive soil community analysis, strand synthesis, soil virome, vime protocol, genomic dna, using superscript iv, resulting d, microbiome extraction, source virome, using klenow polymerase, stranded cdna, superscript iv, quality d

Disclaimer

Abstract

High-Efficiency Double-Stranded cDNA Synthesis from dsRNA Templates
A cost-effective, custom protocol for preparing viral dsRNA for next-generation sequencing using SuperScript IV.

This protocol details a robust and cost-effective method for synthesizing double-stranded cDNA (ds-cDNA) from dsRNA templates. It is optimized for low-input dsRNA, such as that extracted from complex environmental samples (e.g., soil viromes). The protocol uses a two-step process, starting with first-strand synthesis using SuperScript IV Reverse Transcriptase, followed by second-strand synthesis using Klenow Polymerase. The resulting ds-cDNA is suitable for library preparation and next-generation sequencing. This method serves as a reliable alternative to commercial kits.

This protocol is a key downstream component of our **SS-VIME** (Single-Source Virome-Microbiome Extraction) workflow, recently accepted for publication in ***Microbiology Spectrum***. After using the primary **SS-VIME protocol** to co-extract genomic DNA and dsRNA from a single environmental sample, this method is specifically designed to process the purified dsRNA fraction, enabling the characterization of the active RNA virome.

To facilitate high-throughput use, an **Excel-based reagent calculator** is provided in the "Attachments" section. Users simply input the number of samples, and the spreadsheet automatically calculates the required master mix volumes, including a built-in surplus to account for pipetting inaccuracies.

Background
While many commercial kits are available, they can be costly, especially for large-scale projects. This protocol was developed and optimized to provide a high-yield, cost-effective, and reliable workflow for generating high-quality ds-cDNA. It has been successfully used in our lab to prepare sequencing libraries from dsRNA isolated from challenging soil matrices, enabling the study of the active soil virome.

*Note: The associated manuscript, titled **"SS-VIME: A Single-Source Virome-Microbiome Extraction Protocol Toward Comprehensive Soil Community Analysis,"** is currently in press at Microbiology Spectrum. Until the official manuscript DOI is available, please cite this protocol using its protocols.io **DOI**.*

Attachments

XLSX

Double-Stranded cDNA...

13KB

Guidelines

Using the Excel Reagent Calculator

1. **Open the file** and locate the **blue box** labeled "Number of samples?".
2. **Enter the number of samples** you are processing.
3. The spreadsheet will automatically calculate the total volumes needed for each master mix in the corresponding **green boxes**. These calculations include a surplus for one extra reaction (n+0.5) to prevent shortages due to pipetting error.
4. Use the volumes from the **green boxes** to prepare your master mixes.
5. The **purple boxes** indicate the volume of master mix to add to each individual sample.

Materials

Enzymes & Buffers:

- SuperScript™ IV Reverse Transcriptase (Thermo Fisher Scientific)
- Inhibitor, Murine RNase
- Klenow Fragment
- E. coli DNA Ligase
- RNase H
- Random Hexamers (50 µM)
- dNTP Mix (10 mM)
- Dithiothreitol (DTT, 100 mM)
- Nuclease-Free Water

General Labware & Equipment:

- Nuclease-free 0.2 mL PCR tubes or plates
- Pipettes and nuclease-free filter tips
- Thermocycler
- Magnetic rack for bead cleanup
- SPRI beads (e.g., AMPure XP)
- Nuclease-free microfuge tubes

Before start

Using the Excel Reagent Calculator

For efficient and accurate reagent preparation, please use the attached Excel file

1. **Open the file** and locate the **blue box** labeled "Number of samples?".
2. **Enter the number of samples** you are processing.
3. The spreadsheet will automatically calculate the total volumes needed for each master mix in the corresponding **green boxes**. These calculations include a surplus for one extra reaction (n+0.5) to prevent shortages due to pipetting error.
4. Use the volumes from the **green boxes** to prepare your master mixes.
5. The **purple boxes** indicate the volume of master mix to add to each individual sample.

Before You Start

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- Thaw all reagents on ice. Briefly centrifuge all tubes before opening to collect contents at the bottom.
- Keep enzymes on ice or in a cold block at all times.
- When preparing master mixes, calculate the required volume for your number of samples (n) plus at least one extra reaction to account for pipetting error (i.e., calculate for n+0.5). This is reflected in the Excel calculator's "err." column.

Part A: First-Strand cDNA Synthesis

1.1

Step 1: Prepare Denaturation Mix

- In a nuclease-free tube, prepare the following master mix. The volumes below are for a single reaction. Multiply by **n+0.5** for your master mix.

	A	B
	Component	Volume per reaction (µL)
	Random hexamers (50 µM)	1
	dNTPs (10 mM)	1
	Nuclease-free water	1
	Add to each sample (µL)	**3**

1.2 **Step 2: Set up Denaturation Reaction**

- In individual nuclease-free PCR tubes, add **10 µL** of your dsRNA template.
- Add **3 µL** of First-Strand Master Mix #1 to each tube. The total volume should be 13 µL.
- Gently mix by pipetting and centrifuge briefly.

1.3 **Step 3: Incubation - Denaturation**

- Place the tubes in a thermocycler and run the following program:
  - 5 minutes at 95°C

- Hold at 4°C

- Immediately after the program finishes, place the tubes on ice for at least 1 minute.

1.4 **Step 4: Prepare First-Strand Master Mix (RT Mix)**

While the denaturation is running, prepare the following master mix. The volumes below are for a single reaction. Multiply by **n+0.5**.

	A	B
	Component	Volume per reaction (µL)
	SuperScript IV Buffer (5X)	4
	DTT (100 mM)	1
	Inhibitor, Murine RNase (40 U/µL)	0.5
	SuperScript IV RT (200 U/µL)	1
	Nuclease-free water	0.5
	Add to each sample (µL)	**7**

1.5 **Step 5: First-Strand Synthesis**

- Add 7 µL of First-Strand Master Mix #2 to each of your 13 µL reactions on ice. The total volume is now 20 µL.
- Mix gently by pipetting and centrifuge briefly.

1.6 **Step 6: Incubation - First-Strand Synthesis**

- Return the tubes to the thermocycler and run the following program:
  - 10 minutes at 23°C (for primer annealing)
  - 30 minutes at 55°C (for reverse transcription)
  - 10 minutes at 80°C (to inactivate the enzyme)
  - Hold at 4°C
- After the program finishes, place the tubes on ice for at least 1 minute. You can proceed to the next step or store the samples at -20°C.

Part B: Second-Strand cDNA Synthesis

2 **Step 7: Prepare Second-Strand Master Mix**

2.1
- In a nuclease-free tube, prepare the following master mix. The volumes below are for a single reaction. Multiply by n+0.5.
- Pro-Tip: The RNase H volume is very small (0.2 µL). When preparing the master mix, pipette this component carefully and ensure the mix is thoroughly vortexed before aliquoting.

	A	B
	Component	Volume per reaction (µL)
	Klenow 10X buffer (+NAD)	2.5
	dNTP (10 mM)	1
	Klenow polymerase (5 U/µL)	0.5
	E.coli Ligase I (10 U/µL)	1
	Rnase H (5 U/µL)	0.2
	Add to each sample (µL)	**5.2**

2.2 **Step 8: Second-Strand Synthesis**

- To each 20 µL first-strand reaction, add 5.2 µL of Second-Strand Master Mix #3. The final volume will be 25.2 µL.
- Mix gently by pipetting and centrifuge briefly.

2.3 **Step 9: Incubation - Second-Strand Synthesis**

- Return the tubes to the thermocycler and run the following program:
  - 150 minutes (2.5 hours) at 16°C
  - 20 minutes at 75°C (to inactivate enzymes)
  - Hold at 4°C

3

Part C: ds-cDNA Cleanup

4 **Step 10: Bead Purification**

- Perform a standard SPRI bead cleanup to purify the final double-stranded cDNA.

- Use a bead-to-sample ratio of **1.8X**. For a ~25 µL sample, this corresponds to **45 µL** of beads.
- Follow the bead manufacturer's instructions for binding, washing (with 80% ethanol), and elution.
- Elute the purified ds-cDNA in an appropriate volume (e.g., 20-30 µL) of nuclease-free water or buffer. The cDNA is now ready for quantification and library preparation.

Protocol references

SS-VIME: Single-Source Virome-Microbiome Extraction Protocol For simultaneous profiling of viral, bacterial, and fungal communities from a single soil sample dx.doi.org/10.17504/protocols.io.kxygxp64zl8j/v1