First Strand cDNA Synthesis kit

About the Kits

First Strand cDNA Synthesis Kit	40 rxn	69001-3

Description

The First Strand cDNA Synthesis Kit is designed for the preparation of high quality first strand cDNA from cellular RNA templates. The kit contains Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase for superior yields of full-length cDNA. Both Oligo(dT) and Random Hexamer primers are included for a choice of general priming strategies. Alternatively, user-supplied target-specific primers may be used. A small volume (1–2 µl) of the first strand cDNA synthesis reaction is appropriate to use to amplify specific coding regions via PCR with user-supplied primers.

Components

- 4000 U MMLV Reverse Transcriptase
- 200 µl 5X First Strand Buffer
- 100 µl 100 mM DTT
- 50 µl 10 mM dNTP Mix
- 20 µg Oligo(dT) Primer
- 10 µg Random Hexamer Primers
- 1 µg Positive Control RNA, 100 ng/µl
- 10 µl Positive Control Primer, 3' Antisense, 10 pmol/µl
- 10 µl Positive Control Primer, 5' Sense, 10 pmol/µl
- 1.5 ml Nuclease-free Water

Storage

Store Positive Control RNA at –70°C. Store all other components at –20°C. Do not store MMLV Reverse Transcriptase at –70°C.

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General Considerations

Avoiding Ribonuclease Contamination

RNases are ubiquitous in cells and on human skin and efforts should be taken to maintain an RNAse-free laboratory environment. (Blumberg 1987). Ribonucleases present in the workspace and within the RNA sample can rapidly degrade RNA, resulting in poor quality. To avoid RNA degradation, several precautions should be observed:

- All materials and reagents must be sterile and RNase-free. Use sterile disposable pipets, pipet tips, and tubes. Use sterile technique at all times. Use RNAse-free water. (Ehrenberg *et al.* 1976)
- Wear gloves during entire procedure to avoid introducing RNase contaminants to the samples.
- Reserve reagents exclusively for RNA work. Avoid using any reagents that may have been used for other work. Separate any laboratory procedures that require the use of RNase, such as plasmid preps, from RNA work area.
- For gels, use an apparatus that is free of RNase. Particularly avoid any equipment that has been used with plasmid minipreps, since these usually contain copious amounts of RNase. Use RNAse free water for making running buffers.

RNA Isolation

The First Strand cDNA Synthesis Kit can be used with total cellular RNA or fractionated poly(A)$^+$ RNA samples. A widely used method for isolating total RNA has been described by Chomczynski and Sacchi (1987). The Straight A's™ mRNA Isolation System (Cat. No. 69963-3) provides a convenient method for isolating high-quality poly(A)$^+$ RNA directly from tissues or from a total RNA sample. (McCormick and Hammer, 1994).

RNA Template Quality

Although the RNA template can be total RNA, messenger RNA, or viral RNA, subsequent PCR amplification of cDNA usually gives stronger signal when first strand synthesis starts with poly(A)$^+$ mRNA rather than total RNA. Use high purity DNA-free RNA (A_{260}/A_{280} ratio of 1.7 or higher). Gel analysis of mRNA should reveal a smear ranging from 300 bp up to and beyond 4 kbp. The greatest concentration of mRNA will be between 1–2 kbp. Total RNA will also show two rRNA bands.

The quality of the template RNA can also be assessed by setting up a first strand cDNA synthesis using the supplied positive control 3' Primer. The control primers are specific to the housekeeping gene G3PDH (Glyceraldehyde 3-Phosphate Dehydrogenase). The G3PDH gene is expressed in mammalian cells except when treated with cytokine and forbol ester inducers. These G3PDH gene primers can amplify sequence of mouse, rat, swine, or human.

Gel Analysis of the RNA

The RNA can be checked for approximate size and quality by electrophoresis on a non-denaturing gel. RNA samples are denatured in a 90% formamide sample buffer prior to loading. RNAs in this system migrate approximately according to size, but if more accurate size determinations are required, use denaturing electrophoresis. (See TB108 at www.novagen.com/protocol). Always include a control lane of a size standard for measurement and to verify that the apparatus and gel are RNase-free. (e.g. Novagen's Perfect™ RNA Markers, Cat. No. 69946)

1. Pour a 1% agarose gel in 1X TBE (89mM Tris base, 89mM boric acid, 2mM EDTA). Be careful to use clean glassware and high-quality agarose. Use sterile filtered TBE made with RNase-free water. Optional: Include 0.5 g/ml ethidium bromide in the gel.
2. For each lane, mix RNA sample (0.5-2 µg RNA in water or TE) with an equal volume of RNA Sample Buffer (10% sucrose, 90% deionized formamide, 0.05% bromphenol blue, 0.05% xylene cyanol). For Perfect RNA Markers, use 2 µl markers, 8 µl TE, and 10 µl RNA Sample Buffer.
3. Heat at 70°C 3 min, cool to room temperature, and load entire sample onto gel.
4. Run gel at 60 V (up to 10V/cm) in 1X TBE for 1 hour. Monitor progress by the mobility of the dyes in the sample buffer; the bromphenol blue should be approximately 2/3 down the gel.
5. If ethidium bromide was not included in the gel, stain in 0.5 µg/ml ethidium bromide for 10–20 min, destain for 10–20 min. Photograph under UV illumination.

DNase Treatment (Optional)

Some RNA preparations contain significant amounts of genomic DNA, which may be visible as a diffuse high molecular weight band on gels. Such residual DNA in the mRNA template can lead to false signals upon amplification of cDNA. To remove residual DNA treat the RNA solution with RNase-free DNase I (Cat. No. 69182).

1. Adjust RNA solution to 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$.
2. Add 1 U RNase-free DNase I/µg nucleic acid and incubate at 37°C for 10 min
3. Extract once with 1 volume TE-buffered phenol, once with phenol:CIAA, and once with CIAA. For each extraction, add the reagent, vortex for 1 minute, centrifuge at 12,000 x *g* for 1 minute, and transfer the top aqueous phase (containing the RNA) to a fresh tube. TE is 10 mM Tris-HCl pH 8.0, 1 mM EDTA; CIAA is chloroform:isoamyl alcohol (24:1 v/v); phenol:CIAA is a 1:1 v/v mixture of TE-buffered phenol and CIAA.
4. Add 0.5 volume 7.5 M NH_4OAc and 2 volumes 100% ethanol. Incubate at room temperature for 10 min. Centrifuge at 12,000 x *g* at room temperature for 5 min.
5. Remove supernatant, rinse pellet successively with 0.5 ml 70% ethanol and 0.5 ml 100% ethanol. Allow to air dry. Resuspend RNA in an appropriate volume of nuclease-free water. Check the RNA on a gel as described above to verify quality and DNA removal.

Choice of Priming Method for First Strand Synthesis

First strand cDNA synthesis can be primed with a gene-specific primer (not included; supplied by user) or with either the Random Hexamer Primers or Oligo(dT) primer provided in the kit. Oligo(dT) interacts with the 3' poly(A) tail present in most eukaryotic mRNAs and can be used with total DNA or with poly(A)$^+$ RNA (Krug and Berger, 1987). Because of the specificity for mRNA, less optimization is required and results are generally more consistent than with random primers. The recommended amount of Oligo(dT) Primer (0.5 µg) is usually sufficient for priming first strand cDNA synthesis of 0.5–5 µg total RNA or 50–500 ng poly(A)$^+$ RNA. Oligo(dT) priming is convenient when a single first strand synthesis is to be used as template for PCR amplification of multiple gene-specific targets.

A gene-specific primer (provided by the user) can be used to prime first strand synthesis of a particular target sequence. The recommended amount of specific primer (20 pmol) is usually sufficient for priming first strand cDNA synthesis of 0.5–5 µg total RNA or 50–500 ng poly(A)$^+$ RNA. Some adjustment of these concentrations may be needed depending on factors such as target RNA abundance and primer sequence.

Random hexamers are appropriate primers to use when RNA targets are difficult to copy completely with Oligo(dT) primer or gene-specific primer due to secondary structure, when it is necessary to reduce the 3' sequence bias that results from Oligo(dT) priming, or when the target is from an mRNA that lacks a poly(A) tail. The ratio of random primers to RNA can be adjusted to control the average length of cDNA products; higher ratios will produce smaller cDNAs, but should increase the likelihood of copying the target sequence. In general, 50 ng random primers per 5 µg RNA template is appropriate. A high ratio is 250 ng random primers/5 µg RNA. Random hexamers will prime cDNA of all RNAs in a population, not just mRNA. rRNA, a major component of total RNA preps, will also be copied.

The kit includes a Positive Control RNA for use in first strand cDNA synthesis using Random Hexamer Primers, Oligo(dT) Primer, or Positive Control 3' Antisense Primer. Subsequent PCR amplification using the Positive Control 5' Sense Primer and 3' Antisense Primer will produce a 1000 bp product.

cDNA Synthesis

This protocol uses a 3' primer for first strand synthesis of cDNA by MMLV Reverse Transcriptase using an experimental RNA sample as template. The reaction will accommodate samples of 0.5–5 µg total RNA or 50–500 ng poly(A)$^+$ RNA. Use 1 µl of the Control RNA with any of the primers supplied (Random Hexamer Primers, Oligo(dT) Primer, 3' Positive Control Primer) in a control reaction.

First Strand cDNA Synthesis Protocol

1. Combine in a sterile, RNase-free 1.5–ml screw-cap centrifuge tube:

   RNA sample

   0.5–5 µg total RNA
   or 50–500 ng poly(A)$^+$ RNA
   or 1µl Positive Control RNA (100 ng)

   Primer

   0.5 µl (0.5 µg) Oligo(dT) Primer
   or 0.5 µl (50 ng) Random Hexamer Primers
   or 2 µl (20 pmol) Positive Control Primer, 3' Antisense
   or 20 pmol user-supplied gene-specific primer

   X µl Nuclease-free water

   12.5 µl Total volume

2. Heat to 70°C for 10 min. This step denatures RNA secondary structure and may allow more efficient priming and cDNA synthesis.
3. Chill on ice. Spin briefly to collect contents at the bottom of the tube.
4. Add to the 12.5 µl of RNA + primer mixture:

   4 µl 5X First Strand Buffer (5X = 250 mM Tris-HCl 375 M KCl, 15 mM $MgCl_2$, pH 8.3 at 25°C)

   2 µl 100 mM DTT

   1 µl dNTP Mix (10 mM each)

   0.5 µl (100 U) MMLV Reverse Transcriptase

   20 µl Total volume

5. Mix gently by stirring with the pipet tip.
6. If using Random hexamers for priming, incubate at room temperature for 10 min. If using oligo (dT) or gene-specific primer, proceed immediately to step 7.
7. Incubate at 37°C for 60 min. Use the reaction for amplification immediately or store at –20°C.

Amplification

The first strand cDNA synthesis reaction can be used directly as template to PCR-amplify a gene-specific sequence. The example protocols which follow use Nova*Taq*™ Hot Start DNA Polymerase (Cat. No. 71091) or KOD Hot Start DNA Polymerase (Cat. No. 71086). For downstream applications where high accuracy of the product is needed, we recommend KOD Hot Start DNA Polymearase. Some adjustment of these conditions may be needed depending on factors such as target RNA abundance, primer sequences, and choice of thermostable polymerase. Add 1–5 ul of the cDNA synthesis reaction to a mixture containing PCR components and the user supplied gene-specific primers. PCR reagents are not included in the First Strand cDNA Synthesis Kit.

Analyze the reaction products using agarose gel electrophoresis. Usually a 5–10 µl sample of the 50 µl PCR reaction is sufficient to observe the target band. The Positive Control produces a 1000 bp band upon amplification with Positive Control 3' Antisense and 5' Sense Primers.

Amplification using KOD Hot Start DNA Polymerase

1. For each 50 µl reaction, assemble the following in a 0.5–ml PCR tube:

X µl	Nuclease-free water
5 µl	dNTP Mix (2 mM each dATP, dCTP, dGTP, dTTP)
1.5 µl	5' primer (10 pmol/µl)
1.5 µl	3' primer (10 pmol/µl)
5 µl	10X Buffer for KOD Hot Start DNA Polymerse
3 µl	25 mM $MgSO_4$
1 µl	KOD Hot Start DNA Polymerase (1 U/µl)
2 µl	cDNA synthesis reaction
50 µl	total volume

2. Mix gently.

3. Place tubes in thermal cycler.
4. Activate the KOD Hot Start DNA Polymerase with a 2 min 95°C incubation. Cycle as follows: (Appropriate for the 3' and 5' Positive Control Primers. Adjust time or annealing temperature as needed for other gene-specific primers and targets):

   Denature 20 s at 94°C
   Anneal 10 s at 60°C
   Extend 15 s at 70°C

   Repeat for 35 cycles

Amplification using Nova*Taq*™ Hot Start DNA Polymerase

1. For each 50 µl reaction, assemble the following in a 0.5–ml PCR tube:

X µl	Nuclease-free water
1 µl	dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)
0.5 µl	5' primer (10 pmol/µl)
0.5 µl	3' primer (10 pmol/µl) (if needed, see note below)
5 µl	10X Nova*Taq* Hot Start Buffer (10X = 670 mM Tris-HCl, 160 mM $(NH_4)_2SO_4$, 0.1% TWEEN®-20)
3 µl	25 mM $MgCl_2$
0.25 µl	Nova*Taq* Hot Start DNA Polymerase (5 U/µl)
1–5 µl	cDNA synthesis reaction
50 µl	total volume

Note: If using 4–5 ul of a cDNA in which the 3' gene-specific primer was used for first strand cDNA synthesis, additional 3' primer is not needed. However, if Random Hexamer or Oligo(dT) Primers were used to prime first strand synthesis or if small volume of the cDNA is used, 5 pmol of the gene-specific 3' primer should be added to the amplification reaction.

2. Mix gently.
3. Place tubes in thermal cycler.
4. Activate the Nova*Taq* Hot Start DNA Polymerase with a 7 min 95°C incubation. Cycle as follows: (Appropriate for the 3' and 5' Positive Control Primers. Adjust time or annealing temperature as needed for other gene-specific primers and targets):

   Denature 1 min at 94°C
   Anneal 1 min at 50°C
   Extend 2 min at 72°C

   Repeat for 30-40 cycles

   Final extension for 10 min at 72°C

Troubleshooting

The Positive Control RNA and corresponding primers are useful for verifying system performance and identifying sample-related problems. The expected amplification product from the 3 kb Positive Control RNA is 1000 bp. The following table provides a guideline to help troubleshoot the procedure.

Symptom	Possible causes	Controls/Solutions
No amplification products observed	Procedural error	Use Positive Control RNA and appropriate primers to verify performance.
	RNA sample contains inhibitors of cDNA synthesis	Mix 1 µl Positive Control RNA with RNA sample. Perform cDNA synthesis and amplification with Positive Control primers. If reaction fails, inhibitors likely were present in the RNA. Reprecipitate the RNA with 0.1 vol Na acetate and 2.5 vol ethanol, repeat if necessary (rinse pellet with 70% ethanol). Or, perform poly(A)$^+$ selection.
	RNA sample degradation	Check RNA integrity by agarose gel electrophoresis. If RNA appears degraded (prevalence of material less than 1kb in size), reisolate RNA from fresh tissue i .
	Target RNA concentration too low for conditions employed	Increase amount of RNA sample; or use poly(A)$^+$ RNA instead of total RNA. Increase number of cycles for amplification reaction. Switch to Oligo(dT) instead of specific primer for cDNA synthesis.
	Secondary structure in RNA target inhibits cDNA synthesis	Choose a different region for amplification. Perform cDNA synthesis at 50°C. Use random primers for cDNA synthesis.
	Random primer:RNA ratio too high	Optimize ratio.
Unexpected bands observed after amplification	Genomic DNA contamination	Treat RNA prep with DNase.
	Non-specific priming	Increase annealing temperature. Decrease primer and/or RNA concentration. Perform amplification without addition of 5' primer to check for non-specific priming by 3' primer.
	RNA degraded	Check RNA on a gel, repeat RNA prep if necessary.
	Carryover from other reactions	Repeat.

References

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