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I. Description

First Strand cDNA Synthesis Kit 69001-3

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 in addition to user-supplied specific primers. The reaction conditions are compatible with direct addition of primers and other components for amplification of the cDNA product. This kit can be used in conjunction with the Straight A's™ mRNA Isolation System and appropriate PCR reagents* to amplify rare coding regions (*e.g.* Ig variable regions using the Ig-Prime® System).

* The PCR process is covered by patents owned by Hoffmann-La Roche

II. Kit Components

Components (enough for 40 reactions, 20µl each):

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

III. RNA Preparation and Handling

Related Products	Size	Cat. #	Related Products	Size	Cat. #
Straight A's™ mRNA Isolation System plus Separation Stand		69963-3	RNA Markers, 0.36-9.5kb	50µg	69211-3
RNase-free DNase I	1000U	69182-3	Ig-Prime® System		
Oligo(dT) Primer	20µg	69896-3	Human Ig-Prime Kit		69826-3
PCR Markers, 50-2000bp	50 lanes	69278-3	Mouse Ig-Prime Kit		69827-3
Perfect DNA™ Markers, 0.5-12kbp	100 lanes	69002-3	M13mp18 Blunt-End Cloning Kit		69996-3
Perfect RNA™ Markers, 0.2-10kb	50µg	69946-3			



A. RNA Isolation

The First Strand cDNA Synthesis Kit can be used with total or fractionated RNA samples. Stronger amplified signals are usually obtained when starting with poly(A)⁺ RNA for synthesizing specific cDNAs using either the general primers supplied in the kit (oligo(dT) and random hexamers) or specific primers. The Straight A's mRNA Isolation System provides a convenient method for isolating high-quality poly(A)⁺ RNA directly from a variety of tissues (McCormick and Hammer, 1994). A widely used method for isolating total RNA has been described by Chomczynski and Sacchi (1987).

B. Avoiding Ribonuclease Contamination

RNases are ubiquitous in the laboratory and in cells, and precautions should be taken to eliminate the risk of contamination whenever possible (for a discussion see Blumberg 1987). In general, use sterile plastic disposable tubes and pipets, wear gloves, and treat critical solutions with diethyl pyrocarbonate (DEPC). This is done by adding DEPC to 0.1%, stirring vigorously for 10 min, and then heating at 70°C for 1 hour or autoclaving to remove the DEPC. Note that DEPC is an acylating agent that reacts with primary amines and sulfhydryl groups so that reagents such as Tris and DTT cannot be treated directly. In these cases make up the solution with DEPC-treated water and sterile filter or autoclave (do not autoclave DTT). Also note that DEPC should always be used in a fume hood and never added to aqueous solutions containing ammonia, which results in the formation of ethyl carbamate, a potent carcinogen (Ehrenberg *et al.* 1976).

In addition, reserve reagents exclusively for RNA work and store them separately. Avoid using any reagents which may have been used for other work. If possible, separate any laboratory procedures such as plasmid preps which require the use of RNase from your RNA work area.

Quick Gel Analysis of RNA

Although the RNA could be checked by electrophoresis under denaturing conditions, such as on formaldehyde or methylmercury hydroxide agarose gels (for RNA > 1kb), or on formamide or urea polyacrylamide gels, it is usually satisfactory to use the following method, which is faster, safer, and much easier than the others. It is necessary to use an agarose gel apparatus that is RNase-free. Particularly avoid any apparatus that has been used for analysis of plasmid minipreps, since these usually contain copious amounts of RNase. Always run a control lane of a known intact RNA, such as Novagen's Perfect RNA Markers, as size standards and to verify that the apparatus and gel are RNase-free. RNAs run in this system migrate approximately according to size, but these gels should not be used for accurate size determinations.

1. Pour a 1% agarose gel in 1X TBE (89mM Tris base, 89mM boric acid, 2mM EDTA), being careful to use clean glassware and high-quality agarose.
2. Add a sample containing 0.5-2 μ g RNA in water or TE to sample buffer (10% sucrose, 90% deionized formamide, 0.05% bromphenol blue, 0.05% xylene cyanole). Then add 1-2 μ l of 0.1mg/ml ethidium bromide to the sample. Up to 50% of the volume can be the RNA sample, although less is better. The final volume should be no more than 20 μ l.
3. Mix the sample, heat at 60-65°C for 3 min, cool to room temperature, and load the gel.
4. Run the gel at up to 10V/cm in 1X TBE and photograph under UV illumination as for DNA gels. Two prominent bands, the 28s (4.7-5 kb) and 18s (1.95-2.1 kb) ribosomal RNAs, should be clearly visible and distinct.

DNase Treatment (Optional)

Some RNA preparations contain significant amounts of genomic DNA, occasionally visible as a diffuse high molecular weight band on gels, which can lead to false signals upon amplification of cDNA synthesis reaction products. Residual DNA can be removed by treatment with RNase-free DNase I (Cat. # 69182-3) as follows.

1. Adjust RNA solution to 40mM Tris-HCl, pH 7.9, 10mM NaCl, 6mM MgCl₂, 0.1mM CaCl₂.
2. Add 1U RNase-free DNase I/ μ g nucleic acid and incubate at 37°C for 10 min-



- utes.
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 $12,000 \times g$ for 1 minute, and transfer the top aqueous phase (containing the RNA) to a fresh tube. TE is 10mM Tris-HCl pH 8.0, 1mM 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 7.5M NH_4OAc and 2 volumes 100% ethanol and incubate at room temperature for 10 min.
 5. Centrifuge at $12,000 \times g$ at room temperature for 5 min.
 6. Remove supernatant, rinse pellet successively with 0.5ml 70% ethanol and 100% ethanol, allow to air dry, and resuspend RNA in an appropriate volume of DEPC-water. Check the RNA on a gel as described on p. 2 to verify quality and DNA removal.

IV. Choice of Priming Method

Random hexamers and oligo(dT) are provided in the kit. Random primers should be used for RNA samples that are difficult to copy completely with oligo(dT) or specific primer due to secondary structure. 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. Some optimization of the ratio may be necessary for a given target sequence and RNA preparation. In general, 50ng random primers per 5 μg RNA template is appropriate. A high ratio is considered to be 250ng random primers/5 μg RNA.

Since oligo(dT) primers select for poly(A)⁺ RNA, less optimization is required and results are generally more consistent than with random primers. In general, this also holds true for 3' specific primers. The recommended amount of oligo(dT) (0.5 μg) or specific primer (20pmol) is usually sufficient for priming 0.5-5 μg total RNA or 50-500ng poly(A)⁺ RNA. Some adjustment of these concentrations may be needed for specific applications depending on factors such as target RNA abundance and primer sequence.

The kit includes a Positive Control RNA and corresponding primers, which will produce a 1000bp cDNA product using random hexamers, oligo(dT), or Positive Control 3' Antisense primer for first strand synthesis, combined with the Positive Control 5' Sense primer (and 3' Antisense primer, if not used for first strand synthesis) for amplification. In all experiments it is important to include control amplification reactions in which the 5' primer is omitted to test for false positive signals that arise from non-specific priming.

V. cDNA Synthesis

This protocol uses the appropriate 3' primer (random, oligo(dT), or user-specific) for first strand cDNA synthesis from the RNA sample. The reaction will accommodate from 0.5-5 μg total RNA or 50-500ng poly(A)⁺ RNA. Use 1 μl of the Control RNA with any of the primers supplied (random, oligo(dT), 3' Control Primer) in a control reaction. When using random primers, a 10 min incubation at room temperature is carried out after MMLV RT addition to allow efficient priming, followed by incubation at 37°C for 60 minutes for cDNA synthesis.



First Strand cDNA Synthesis Kit

- Combine in a sterile, RNase-free 1.5ml screw-cap microcentrifuge tube:
RNA sample
0.5-5µg total RNA
or 50-500ng poly(A)⁺ RNA
or 1µl Positive Control RNA (100ng)
Primer
0.5µl (0.5µg) Oligo(dT)
or 0.5µl (50ng) Random Hexamers
or 2µl (20pmol) Positive Control Primer, 3' Antisense
or 20pmol user-specific primer
xµl Nuclease-free water
12.5µl Total volume
- Heat to 70°C for 10 minutes. This step helps to alleviate RNA secondary structure and may allow more efficient priming and cDNA synthesis.
- Chill quickly on ice.
- Microcentrifuge briefly to collect contents at the bottom of the tube.
- Add the remaining components for first strand cDNA synthesis:
4µl 5X First Strand Buffer (5X = 250mM Tris-HCl pH 8.3 at 25°C, 375mM KCl, 15mM MgCl₂)
2µl 100mM DTT
1µl dNTP Mix (10mM each)
xµl Nuclease-free water
0.5µl (100U) MMLV Reverse Transcriptase
20µl Total volume
- Mix gently by stirring with the pipet tip. *When using Random Hexamers, incubate at room temperature for 10 min before placing at 37°C.* Incubate at 37°C for 60 min. The reaction may be used directly for amplification or stored at -20°C.

VI. Amplification

The cDNA products (5µl samples) can be amplified directly by adding them to a mixture containing PCR components and the appropriate 5' primer. Residual 3' specific primers carried over from the first strand reaction eliminate the need for additional 3' primer addition. However, if Random Hexamers or Oligo(dT) was used to prime first strand synthesis, 5pmol of a specific 3' primer should be added to the amplification reaction. This protocol uses 5µl samples of the cDNA reactions in a final volume of 50µl.

- Make a master reaction mix as follows:
Per reaction:
xµl Nuclease-free water
1µl 10mM dNTP mix (supplied by user)
5µl 10X buffer (10X = 100mM Tris-HCl pH 8.8 at 25°C, 500mM KCl, 15mM MgCl₂, 1% Triton X-100) (supplied by user)
5pmol 5' primer, 10pmol/µl; for Positive Control reaction, add 0.5µl (5pmol) Positive Control Primer, 5' Sense
5pmol 3' primer, if necessary (see above)
0.25µl (1.25U) AmpliTaq DNA polymerase (Perkin-Elmer)
45µl Total volume
Mix together the above components in a single tube using amounts corresponding to the number of reactions desired. (It is convenient to multiply the amounts by X.5, where X is the number of reactions, in order to account for pipetting losses.)
- Add 5µl of the first strand cDNA reactions to 0.5ml sample tubes. Add 45µl of master mix, mix gently, add 2 drops of mineral oil, cap the tubes and put the samples in a thermal cycler (Perkin-Elmer). For a "hot start" PCR (which may reduce false priming), first place the tubes containing the cDNA in the thermal cycler set at 80°C and then quickly add the master mix and oil. Process up to 4 samples at once to avoid losses from evaporation (reactions can be kept at



80°C under oil until all samples are ready). After all samples are capped start the PCR cycling by ramping directly to the first denaturation time period.

3. Cycle for 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, with a final extension at 72°C for 6 min. Amplification of a specific sequence depends on the quantity of RNA used and abundance of the target RNA. Generally 30-45 cycles are required for satisfactory amplification under these conditions.
4. Analyze the reaction products using agarose gel electrophoresis. Usually a 5-10µl sample is sufficient to observe the target band. The Positive Control produces a 1000bp band upon amplification with Positive Control 3' Antisense and 5' Sense Primers.

Note:

- a. Novagen's primers are designed and sold for use in the Polymerase Chain Reaction (PCR) process covered by patents owned by Hoffmann-La Roche. Use of the PCR process requires a license. A license for research may be obtained by purchase and use of authorized reagents and DNA thermal cyclers from The Perkin-Elmer Corporation or by otherwise negotiating a license with Perkin-Elmer.

VII. Troubleshooting

The Positive Control RNA and corresponding primers are useful for verifying system performance and isolating sample-related problems. The expected amplification product from the 3kb Positive Control RNA is 1000bp. 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 1µl Positive Control RNA with RNA sample for cDNA synthesis and amplification with Positive Control primers. If reaction fails, inhibitors likely present. Reprecipitate 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 formaldehyde agarose gel electrophoresis; reisolate from fresh tissue if RNA appears degraded (prevalence of material less than 1kb in size).
	Target RNA concentration too low for conditions employed	Increase amount of RNA sample; use poly(A) ⁺ RNA in place of total RNA. Increase number of cycles for amplification reaction. Switch to oligo(dT) instead of specific primer.
	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.
Unexpected bands observed after amplification	Random primer:RNA ratio too high	Optimize ratio.
	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	Be careful!



VIII. References

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