2nd Strand cDNA Synthesis Protocol using the Template Switching RT Enzyme Mix (NEB #M0466)

Overview

The following protocol can be used to synthesize ds cDNA that covers the full 5′ transcription start site when the 5′ sequence of the transcript is unknown, providing an advantage over the Gubler and Hoffman 2nd Strand cDNA Synthesis method [1, 2]. This protocol contains two steps. In the first step, template switching reverse transcription generates cDNAs with a universal sequence of choice attached to the 3′ end of cDNA, mediated by the template switching oligo (TSO). In the second step, RNA is hydrolyzed and 2nd strand cDNA is subsequently synthesized by primer extension using the TSO as a primer.

Sample Recommendations:

To synthesize double stranded cDNA containing transcription start sites high quality intact RNA is required, usually in the range of 100 ng - 5 µg. The RNA sample should be free of salts (e.g., Mg^{2+}, guanidinium), divalent cation chelating agents (e.g., EDTA, EGTA, citrate) or organics (e.g., phenol, ethanol). If an excess amount of genomic DNA is present in RNA samples, an optional DNase I treatment can be performed. Inactivate or remove DNase I after treatment.

Reaction Preparation

1. Briefly centrifuge the Template Switching RT Enzyme Mix to collect the solution to the bottom of the tube, then place on ice.
2. Thaw the Template Switching RT Buffer at room temperature completely. Vortex and centrifuge briefly to collect the solution to the bottom of the tube, then place on ice.

cDNA Synthesis and Template Switching

Please note that the volume needed for each reagent is based on a final cDNA Synthesis and Template Switching reaction volume of 10 µl. If desired, the reaction can be scaled up proportionally, while observing the RNA input amount limit, to a final volume of 20 µl.

Primer Annealing for 1st Strand Synthesis

1.1 To anneal the RT primer with the RNA templates, in a 0.2 ml nuclease free PCR tube, prepare the reaction as follows (on ice):

Reagent	Volume	Final Concentration
RNA	Up to 4 µl	100 ng – 5 µg

Reagent	Volume	Final Concentration
Oligo(dT)$_{40}$VN or GSP* (10 µM)	1 µl	1 µM
dNTP (10 mM)	1 µl	1 mM
Nuclease-free Water	Variable	-
Total Volume	6 µl	-

*GSP: gene-specific primer

Mix thoroughly by gently pipetting up and down at least 10 times, then centrifuge briefly to collect the solution to the bottom of the tube.

1.2 Incubate for 5 minutes at 70°C in a thermocycler with the lid temperature set at ≥85°C, then hold at 4°C until next step.

2.Reverse Transcription (RT) and Template Switching

2.1 During the primer annealing reaction, vortex Template Switching RT Buffer briefly followed by a quick spin to collect the solution to the bottom of the tube, then prepare the RT reaction mix as follows (adding enzyme mix last):

Reagent	Volume	Final Concentration
Template Switching RT Buffer (4X)	2.5 µl	1X
Template Switching Oligo (TSO) (75 µM)	0.5 µl	3.75 µM
Template Switching RT Enzyme Mix (10X)	1 µl	1X
Total Volume	4 µl	-

Mix thoroughly by pipetting up and down for 10 times, then centrifuge briefly to collect the solution to the bottoms of the tube.

2.2 Combine 4 µl RT reaction mix (above) with 6 µl of the annealed mix from step 1.2, mix well by gently pipetting up and down at least 10 times, then centrifuge briefly to collect solution to the bottom of the tube.

2.3 Incubate the 10 µl combined reaction in a thermocycler with the following steps:

90 minutes at 42°C
5 minutes at 85°C
Hold at 4°C

If not proceeding to the 2nd strand cDNA Synthesis step immediately, samples can be stored at 4°C overnight or at -20°C for up to one week.

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2nd Strand cDNA Synthesis

RNA Template Degradation and Primer Extension

1. Assemble the 2nd strand cDNA synthesis reaction (on ice) by adding the following components into the cDNA product from step 2.3:

Components	Volume	Final Concentration
Template switching cDNA product (from step 2.3)	10 µl	-
Q5 Hot Start High Fidelity Master Mix (NEB #M0494)	50 µl	1X
E.coli RNase H (5U/µl)	5 µl	0.25 U/µl
H_2O	35 µl	-
Total Volume	100 µl	-

Mix gently and then centrifuge briefly to collect solutions to the bottom of tubes.

2. Incubate in a thermocycler, with lid temperature set at ≥ 100°C and perform RNA hydrolysis followed by primer extension as follows:

Step	Temperature	Time (minutes)
RNA template hydrolysis	37°C	15
Initial denaturation	95°C	1
Primer extension	65°C	10
Hold	4°C	∞

If not proceeding to the next step immediately, samples can be stored at 4°C overnight or at -20°C for up to one week.

3.The ds cDNA can be further purified with Monarch® Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130) or with AMPure/SPRIselect beads (Beckman Coulter) following manufacturer recommendations.

General Guidelines:

1. RNA template

Sufficient amount of intact RNA, in the range of 100 ng – 5 µg, is required. The typical ds cDNA yield is 30-60 ng per 100 ng of mRNA input and 200-400 ng for 5 µg of intact UHR total RNA.

2. RT primer

The RT primer could be either gene-specific or poly (dT) for poly(A) containing RNA templates. If poly(dT) is used, we recommend $d(T)_{40}VN$.

3. TSO

The TSO functions as the primer for 2nd strand cDNA synthesis by primer extension, so 3´ modifications that may prevent extension should be avoided. An rU containing TSO may cause inhibition if an archeal family BDNA polymerase (e.g., Q5) is used for primer extension. If cloning is to be performed after 2nd strand synthesis, 5´ modifications that may prevent ligations should be avoided.

4. Primer for 2nd strand cDNA synthesis

No additional primer is needed. The TSO will function as a primer for 2nd strand synthesis by primer extension.

5. 2nd strand cDNA synthesis by primer extension

The RT reaction does not need to be purified prior to 2nd strand cDNA synthesis. If a cDNA cleanup step is desired, we recommend AMPure XP or SPRIselect bead at a 1:2.5 sample to bead volume ratio. Please follow the manufacturer's recommendations for a detailed sample clean up procedure.

6. 2nd strand cDNA synthesis products

The ds cDNA products have blunt ends. A cleanup step is generally needed prior to any downstream applications. The cleanup can be done using the Monarch® Spin PCR & DNA Cleanup Kit (NEB #T1130) or AMPure XP or SPRI select bead at a 1:1 bead to sample volume ratio. If cloning is to be performed, we recommend the NEB® PCR Cloning Kit (NEB #E1202).

References:

1. Okayamam H. and Berg, P. (1982) Mol. Cell Biol. 2 (2), 161-170
2. Gubler. R and Hoffman, BJ. (1983) Gene 25(2-3): 263-269