Protocol for Use with Total RNA - NEBNext mRNA First Strand Synthesis Module (E7525)

Materials Required but not Supplied

NEBNext® RNA 第一链合成模块
如果使用此模块进行定向 RNA 测序，则需要使用放线菌素 D（Sigma #A1410，在二甲基亚砜 [DMSO] 中溶解至 5 µg/µl）。详细信息，请参阅操作说明。

磁力架或磁力板（例如：NEBNext® 磁性分离架，NEB #S1515S；Alpaqua ® 96S 超磁性分离板，#A001322；或同等器材）

Overview

Symbols

⚠ *This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.*

Note: Follow steps in Protocol (A) if starting material is total RNA. Perform mRNA isolation, fragmentation and priming using the NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB #E7490). If starting material is purified mRNA or ribosomal depleted RNA, proceed to (B).

Protocol A for Use with Total RNA

Starting Material: Total RNA (DNA free) (10 ng–1 µg) purified mRNA (10–100 ng), or ribosomal depleted total RNA (10–100 ng).

RNA Purity: The RNA sample should be free of DNA, salts (e.g., Mg^{2+}, or guanidinium salts), divalent cation chelating agents (e.g., EDTA, EGTA, citrate), or organics (e.g., phenol and ethanol).

1. Preparation of First Strand Reaction Buffer and Random Primer Mix

Prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) as follows in a nuclease-free tube:

Component	Volume
NEBNext First Strand Synthesis Reaction Buffer	8 µl
NEBNext Random Primers	2 µl
Nuclease-free water	10 µl
Total Volume	**20 µl**

2. mRNA Isolation, Fragmentation and Priming Starting with Total RNA

2.1. Dilute the total RNA with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.

2.2. To wash the Oligo dT Beats, add the following into a 1.5 ml nuclease-free tube. If preparing multiple libraries, beads for up to 10 samples can be added to a single 1.5 ml tube for subsequent washes (use magnet NEB #S1506 for 1.5 ml tubes). The purpose of this step is to bring the beads from the storage buffer into the binding buffer. The 2X Binding Buffer does not have to be diluted for this step.

Component	**Volume Per One Library**
Oligo dT Beads $d(T)_{25}$	20 µl
RNA Binding Buffer (2X)	100 µl
Total Volume	**120 µl**

2.3. Wash the beads by pipetting up and down six times.

2.4. Place the tube on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

2.5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

2.6. Remove the tube from the magnetic rack.

2.7. Add 100 µl RNA Binding Buffer (2X) to the beads and wash by pipetting up and down six times. If preparing multiple libraries, add 100 µl RNA Binding Buffer (2X) per sample.

2.8. Place the tubes on the magnet and incubate at room temperature until the solution is clear (~2 minutes).

2.9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

2.10. Add 50 µl RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing multiple libraries, add 50 µl RNA Binding Buffer (2X) per sample. This first binding step removes most of the non target RNA.

2.11. Add 50 µl beads to each RNA sample from Step 2.1. Mix thoroughly by pipetting up and down six times.

2.12. Place the tube in a thermocycler and close the lid. Heat the sample at 65°C for 5 minutes and cool to 4°C with the heated lid set at ≥ 75°C to denature the RNA and facilitate binding of the mRNA to the beads.

2.13. Remove the tube from the thermocycler when the temperature reaches 4°C.

2.14. Mix thoroughly by pipetting up and down six times. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.

2.15. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

2.16. Remove and discard all of the supernatant. Take care not to disturb the beads.

2.17. Remove the tube from the magnetic rack.

2.18. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.

2.19. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

2.20. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

2.21. Remove the tube from the magnetic rack.

2.22. Repeat steps 2.18–2.21.

2.23. Add 50 µl of Tris Buffer (provided in NEB #E7490 kit) to each tube. Gently pipette up and down 6 times to mix thoroughly.

2.24. Place the tube on the thermocycler. Close the lid and heat the samples at 80°C for 2 minutes, then cool to 25°C with the heated lid set at ≥ 90°C to do the first elution of the mRNA from the beads.

2.25. Remove the tube from the thermocycler when the temperature reaches 25°C.

2.26. Add 50 µl of RNA Binding Buffer (2X) to the sample to allow the mRNA to re-bind to the beads. Mix thoroughly by gently pipetting up and down six times.

2.27. Incubate the tube at room temperature for 5 minutes.

2.28. Place the tube on the magnetic rack at room temperature until the solution is clear (~2 minutes).

2.29. Remove and discard the supernatant from the tube. Take care not to disturb the beads.

2.30. Remove the tube from the magnetic rack.

2.31. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.

2.32. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

Note: It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.

2.33 Place the tube on the magnet at room temperature until the solution is clear (~2 minutes).

2.34. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads that contains the mRNA.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip, remove all of the wash buffer. (Caution: Do not disturb beads that contain the mRNA). Avoid letting the beads dry out before adding elution buffer.

2.35. Remove the tube from the magnetic rack. 6 Note: The next step provides a fragmentation incubation time resulting in an RNA insert size of ~ 200 nt.

2.36. To elute the mRNA from the beads and fragment, add 15.5 µl of the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1. Pipette up and down six times to resuspend the beads.

2.37 Incubate the sample in a thermocycler with the heated lid set at 105°C as follows:

15 minutes at 94°C

Hold at 4°C*

*Immediately transfer the tube to ice for 1 minute as soon as it is cool enough to handle (~65°C)

2.38. Quickly spin down the tube in a microcentrifuge to collect the liquid from the sides of the tube and place on the magnet right away until the solution is clear (~1-2 minutes).

2.39. Collect the fragmented mRNA by transferring 13.5 µl of the supernatant to a nuclease-free 0.2 ml PCR tube.

Note 1: If the supernatant volume recovered is less than 13.5 µl for any reason, bring the volume up to 13.5 µl by adding the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) prepared in Step 1 and continue with the protocol.

Note 2: Avoid transferring any of the magnetic beads.

2.40. Place the tube **on ice** and proceed directly to First Strand cDNA Synthesis.

3. First Strand cDNA Synthesis

Note: If you are performing first strand synthesis as part of a directional RNA sequence workflow, it is recommended to add Actinomycin D to the reaction.

3.1. ⚠ *Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.*

Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) are expected to be stable for at least a month at -20°C.

To the fragmented and primed mRNA (10 µl from Step 2.40) add the following components:

3.2A. **Non-Directional Reaction Step Up:**

Component	**Volume**
(pink) Murine RNase Inhibitor	0.5 µl
(pink) ProtoScript II Reverse Transciptase	1 µl
Nuclease free water	5 µl
Final Volume	**20 µl**

3.2B. **Directional Reaction Step Up:**

Component	**Volume**
(pink) Murine RNase Inhibitor	0.5 µl
(pink) ProtoScript II Reverse Transciptase	1 µl
Actinomycin D (0.1 µg/µl)	5 µl
Final Volume	**20 µl**

3.3 Incubate the sample in a preheated thermocycler with the heated lid set at ≥ 80°C as follows:

10 minutes at 25°C
50 minutes at 42°C
15 minutes at 70°C
Hold at 4°C

3.4. Immediately, perform second strand synthesis reaction, using NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (NEB #E6111), or NEBNext Ultra II Directional RNA Second Strand Synthesis Module (NEB #E7550).