NEBNext® High Input Poly(A) mRNA Isolation Module

NEB #E3370S

24 reactions

Version 1.0_1/23

Table of Contents

The NEBNext High Input Poly(A) mRNA Isolation Module Includes

The volumes provided are sufficient for preparation of up to 24 reactions.
All reagents should be stored at 4°C.

NEBNext High Input Oligo d(T)$_{25}$ Beads

NEBNext RNA Binding Buffer (2X)

NEBNext Wash Buffer

NEBNext Tris Buffer

Nuclease-free Water

Required Materials Not Included:

0.2 ml RNase-free tube, for example TempAssure® PCR flex-free 8-tube strips (USA Scientific® #1402-4708)

Magnetic Rack (NEB#S1515S, Alpaqua®, cat. #A001322, or equivalent)

1.5 ml Microcentrifuge tube and NEB #S1506 Magnet stand or equivalent (for bulk-washing beads)

Thermal cycler or heat block

Bioanalyzer® (Agilent Technologies, Inc.) or similar instrument and consumables

Description

The NEBNext High Input Poly(A) mRNA Isolation Module is designed to isolate intact poly(A) RNA from high inputs (5-50 µg) of previously isolated total RNA. The technology is based on the coupling of oligo d(T)$_{25}$ to paramagnetic beads, which is then used as the solid support for the direct binding of poly(A) RNA. Thus, the procedure permits the manual processing of multiple samples and can be adapted for automated high-throughput applications. Additionally, magnetic separation technology permits elution of intact mRNA in small volumes, thereby eliminating the need for precipitation of the poly(A) transcripts in the eluent. Intact poly(A) RNA which is representative of the mRNA population of the original sample can be obtained in approximately one hour.

Application

Isolation of poly(A) RNA transcripts from high inputs (5-50 µg) of total RNA for RNA library preparation and sequencing.

Isolate Poly-Adenylated RNA Using the NEBNext High Input Poly(A) mRNA Isolation Module (NEB #E3370S)

The protocol has been optimized using high quality Universal Human Reference Total RNA (Agilent Technologies, Inc.).

RNA Sample Requirements:

The RNA sample should be free of excess salts (e.g., Mg^{2+} or guanidinium salts), divalent cation chelating agents (e.g., EDTA or EGTA), or organics (e.g., phenol or ethanol). RNA must be free of DNA. gDNA is a common contaminant from RNA preps. It may be carried over from the interphase of organic extractions or when the silica matrix of solid phase RNA purification methods is overloaded. If the total RNA sample may contain gDNA contamination, treat the sample with DNase I to remove all traces of DNA (DNase is not provided in this kit). After treatment with DNase I the enzyme should be removed from the sample. Any residual activity of the DNase I will degrade the oligos necessary for the enrichment. DNase I can be removed from the extraction using phenol/chloroform extraction and ethanol precipitation or silica column methods such as the Monarch® RNA Cleanup Kit (NEB #T2030). Contaminating DNA can cause inaccurate RNA quantification and impede proper rRNA removal.

RNA Integrity:

Assess the quality of the Input RNA by running the RNA sample on an Agilent Bioanalyzer RNA 6000 Nano/Pico Chip. For Poly(A) mRNA enrichment, high quality RNA with a RIN score > 7 is required.

Note: For best results, keep all the reagents used during the poly(A) isolation on ice when not in use.

Starting Material: 5 –50 µg* of DNA-free total RNA.

* For inputs above 50 µg, volumes can be increased proportionally. For inputs less than 5 µg, use NEBNext Poly(A) mRNA Isolation Module (NEB #E7490).

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 until step 11.
2. To wash the High Input Oligo $d(T)_{25}$ Beads, add the following to a 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.

   Note: Vortex all buffers before use. Do not vortex RNA or beads. Mix the beads well before using by flicking or inverting the tube to ensure a homogeneous suspension.

COMPONENT	VOLUME PER ONE LIBRARY
High Input Oligo $d(T)_{25}$ Beads	20 µl
RNA Binding Buffer (2X)	100 µl
Total Volume	**120 µl**

3. Mix by pipetting the beads up and down ten times.
4. Place the tube on the magnetic rack at room temperature for 2 minutes or until the solution is clear.
5. Remove and discard the supernatant from the tube. Take care not to disturb the beads.
6. Remove the tube from the magnetic rack.
7. Add 100 µl RNA Binding Buffer (2X) to the beads and wash by pipetting up and down ten times. If preparing multiple libraries, add 100 µl RNA Binding Buffer (2X) per sample. The Binding Buffer does not have to be diluted.
8. Place the tubes on the magnet and incubate at room temperature until the solution is clear (~2 minutes).
9. Remove and discard the supernatant from the tube. Take care not to disturb the beads.
10. Remove tubes from magnet and add 50 µl RNA Binding Buffer (2X) to the beads and mix by pipetting up and down until beads are homogenous. If preparing beads in bulk, add 50 µl RNA Binding Buffer (2X) per sample.
11. Combine 50 µl washed beads in RNA Binding Buffer (2X) with each 50 µl RNA sample from Step 1. Mix thoroughly by pipetting up and down ten times. This first binding step removes most of the non-target RNA.

12. To denature the RNA and facilitate binding of the mRNA to the beads, place the tubes on the thermal cycler and heat the sample at 65°C for 5 minutes and cool to 4°C with the heated lid set at ≥ 75°C.
13. Remove tubes from the thermal cycler when the temperature reaches 4°C.
14. Resuspend the beads on the bench. Pipette up and down slowly ten times to mix thoroughly.
15. Place the tubes on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads.
16. Resuspend the beads. Pipette up and down slowly ten times to mix thoroughly.
17. Incubate for 5 more minutes on the bench at room temperature to allow the RNA to bind to the beads.
18. Place the tubes on the magnetic rack at room temperature for 2 minutes or until the solution is clear to separate the poly(A) RNA bound to the beads from the solution.
19. Remove and discard the supernatant. Take care not to disturb the beads.
20. Remove the tubes from the magnetic rack.
21. Wash the beads by adding 200 µl of Wash Buffer to remove unbound RNA. Pipette the entire volume up and down ten times to mix thoroughly.

    Note: Bead clumping may occur during washes for samples with high poly(A) RNA content. This can be reduced with additional mixing, but has not been observed to affect RNA quality.
22. Place the tubes on the magnetic rack at room temperature for 2 minutes or until the solution is clear.
23. Remove and discard all the supernatant from each well of the tube. Take care not to disturb the beads.
24. Remove the tubes from the magnetic rack.
25. Repeat Steps 21-24 once, for a total of two washes.
26. Add 50 µl of Tris Buffer (provided in NEB #E3370S kit) to each tube. Gently pipette the entire volume up and down ten times to mix thoroughly.
27. Place the tubes on the thermal cycler. Close the lid and heat the sample 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.
28. Remove the tubes from the thermal cycler when the temperature reaches 25°C.
29. Add 50 µl of RNA Binding Buffer (2X) to each sample to allow the RNA to re-bind to the same beads. Gently pipette the entire volume up and down ten times to mix thoroughly. This second binding step increases the specificity of mRNA binding.
30. Incubate the tubes on the bench at room temperature for 5 minutes.
31. Resuspend the beads. Pipette up and down slowly ten times to mix thoroughly.
32. Incubate the tubes on the bench at room temperature for 5 more minutes to allow the RNA to bind to the beads.
33. Place the tubes on the magnetic stand at room temperature for 2 minutes or until the solution is clear.
34. Remove and discard the supernatant from each tube. Take care not to disturb the beads.
35. Remove the tubes from the magnetic rack.
36. Wash the beads once with 200 µl of Wash Buffer. Gently pipette the entire volume up and down ten times to mix thoroughly. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.
37. Place the tubes on the magnetic rack at room temperature for 2 minutes or until the solution is clear.
38. Remove and discard the supernatant from each tube. Take care not to disturb the beads.

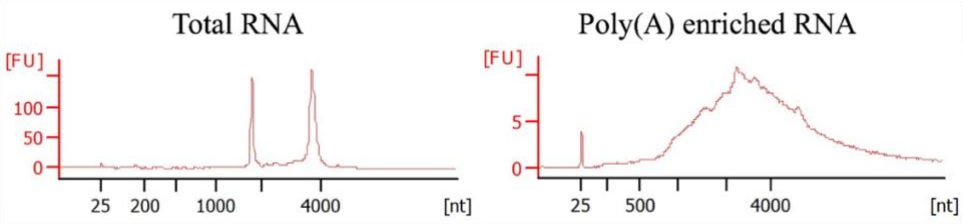
    Note: It is important to remove all of the supernatant to successfully use the RNA in downstream applications. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip remove any remaining wash buffer. (Caution: Do not disturb the beads that contain the mRNA). Avoid letting the beads dry out before adding elution buffer.
39. Remove the tubes from the magnetic rack.
40. Elute the mRNA from the beads by adding 17 µl of the Tris Buffer, mix by pipetting ten times and incubating the sample at 80°C for 2 minutes, then cool to 25°C to elute the poly(A) RNA from the beads.

41. Immediately, place the tubes on the magnetic rack for 2 minutes or until the solution is clear.

42. Collect the purified mRNA by transferring 15 µl of the supernatant to a clean nuclease-free PCR Tube. Place on ice if using immediately. Alternatively, the sample can be placed at –80°C for long term storage.

43. (Optional) Assess the yield and the size distribution of the purified mRNA. Run 1 µl on a Bioanalyzer. You may need to dilute your sample before loading.

Figure 1: Example of RNA distribution on a Bioanalyzer before and after Poly(A) enrichment.



Checklist:

[_] 1. Dilute RNA to 50 µl in 0.2 ml tube

[_] 2. New 0.2 ml tube: aliquot 20 µl NEBNext High Input Oligo $d(T)_{25}$ Beads

[_] 3. Add 100 µl of RNA Binding Buffer (2X) and mix ten times

[_] 4. Place tube on magnet for 2 minutes

[_] 5. Remove and discard supernatant

[_] 6. Remove tube from magnet

[_] 7. Add 100 µl of RNA Binding Buffer (2X) and pipet mix ten times

[_] 8. Place tube on magnet for 2 minutes

[_] 9. Remove and discard supernatant

[_] 10. Remove tube from magnet, add 50 µl RNA Binding Buffer (2X), pipet mix

[_] 11. Combine 50 µl resuspended beads with50 µl total RNA sample and pipet mix

[_] 12. Heat the sample at 65°C for 5 minutes, then hold at 4°C

[_] 13. Remove tubes when temperature is 4°C

[_] 14. Resuspend beads by pipet mixing slowly ten times

[_] 15. Incubate tubes on bench for 5 minutes

[_] 16. Resuspend beads by pipet mixing slowly ten times

[_] 17. Incubate tubes 5 more minutes on bench

[_] 18. Place tubes on magnet for 2 minutes

[_] 19. Remove and discard supernatant

[_] 20. Remove tube from magnet

[_] 21. Wash beads with 200 µl Wash Buffer by pipet mixing ten times

[_] 22. Place tubes on magnet for 2 minutes

[_] 23. Remove and discard supernatant

[_] 24. Remove tubes from magnet

[_] 25. Repeat Steps 21 [_], 22 [_], 23 [_] and 24 [_]

[_] 26. Add 50 µl Tris to tubes and mix by pipetting ten times

[_] 27. Place tubes on thermal cycler, heat at 80°C for 2 minutes, then cool to 25°C

[_] 28. Remove tubes from thermal cycler when temperature is 25°C

[_] 29. Add 50 µl RNA Binding Buffer (2X) to same beads in tubes and pipet mix ten times

[_] 30. Incubate tubes at room temperature for 5 minutes

[_] 31. Resuspend beads by pipet mixing slowly ten times

[_] 32. Incubate tubes on bench for 5 more minutes

[_] 33. Place tubes on magnet for 2 minutes

[_] 34. Remove and discard supernatant

[_] 35. Remove tubes from magnet

[_] 36. Wash beads with 200 µl of Wash Buffer by pipet mixing ten times

[_] 37. Place tubes on magnet for 2 minutes

[_] 38. Remove and discard supernatant

[_] 39. Remove tubes from magnet

[_] 40. Add 17 µl Tris Buffer to elute mRNA, pipet mix, and incubate at 80°C for 2 minutes, cool to 25°C

[_] 41.Immediately put on magnet for 2 minutes

[_] 42. Transfer 15 µl of the supernatant (containing the purified mRNA) into a clean nuclease-free PCR Tube. Place on ice if using immediately. Alternatively, the sample can be placed at –80°C for long term storage.

[_] 43. (Optional) Run mRNA on a Bioanalyzer RNA Chip.

Kit Components

NEB #E3370S Table of Components

NEB #	PRODUCT	VOLUME
E3371A	NEBNext High Input Oligo d(T)$_{25}$ Beads	0.480 ml
E7492A	NEBNext RNA Binding Buffer (2X)	7.2 ml
E7493A	NEBNext Wash Buffer	28.8 ml
E7496A	NEBNext Tris Buffer	6.0 ml
E7495A	Nuclease-free Water	1.2 ml

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	01/23

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