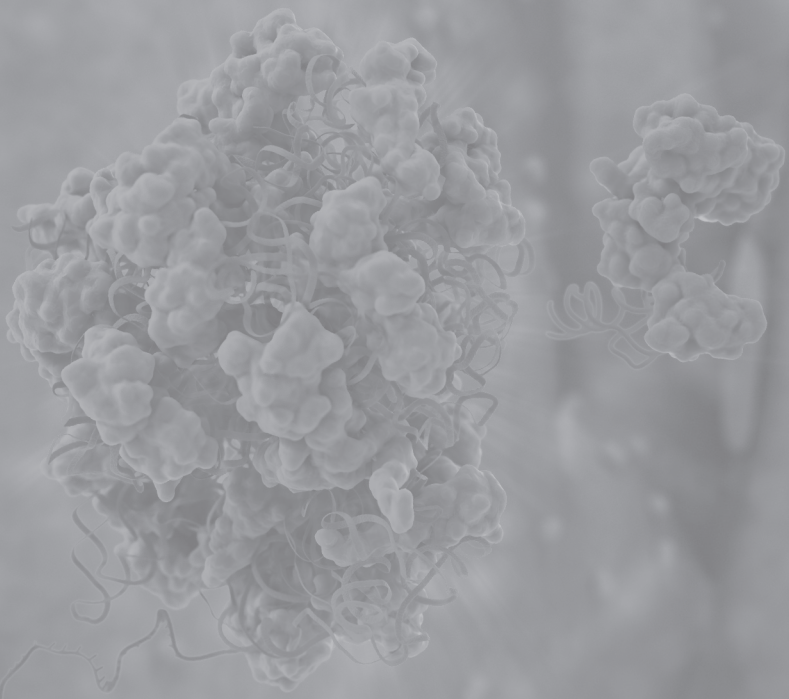
RNA ANALYSIS

polyA Spin™ mRNA Isolation Kit

Instruction Manual



NEB #S1560S
8 isolations
Version 1.1 5/11

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be INSPIRED
drive DISCOVERY
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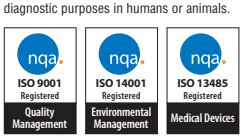

Table of Contents:

Kit Components:

The polyA Spin™ mRNA Isolation Kit Includes:

Wash Buffer
20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA ..... 15 ml

Elution Buffer
20 mM Tris-HCl (pH 7.5), 1 mM EDTA ..... 10 ml

Low Salt Buffer
10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA ..... 3.5 ml

5 M NaCl ..... 1.0 ml

3 M NaAc ..... 0.5 ml

Glycogen Solution
10 mg/ml ..... 200 µl

8 prepacked microcentrifuge tubes containing oligo $(dT)_{25}$-cellulose beads in storage buffer.

10 microcentrifuge spin columns for isolation of poly(A)$^+$ RNA.

8 sterile microcentrifuge tubes for the deposit of isolated poly(A)$^+$ RNA.

Instruction Manual

Introduction:

The NEB polyA Spin™ mRNA Isolation Kit is designed as a rapid and convenient alternative to traditional column chromatography for the isolation of full-length poly(A)$^+$ messenger RNA (mRNA) from cytoplasmic or 'total' RNA isolated from eukaryotic tissue and cells. Poly(A)$^+$ RNA selection is made by affinity chromatography using pre-packed oligo $(dT)_{25}$-cellulose beads. The matrix consists of oligo $(dT)_{25}$ covalently coupled to a cross-linked cellulose bead. The large porous surface area of the bead permits high density of ligand as well as rapid hybridization kinetics during poly(A)$^+$ RNA selection. Intact messenger RNA can be isolated in as little as forty-five minutes from multiple cell lysates or samples of 'total' RNA by spin chromatography in a microcentrifuge. Sufficient reagents are provided for the isolation and subsequent precipitation of poly(A)$^+$ RNA from eight samples of total RNA of as much as 1.0 mg or 5 x 10^7 cells each. RNA isolated can be used for *in vitro* translation experiments, the preparation of cDNA libraries, Northern analysis, subtractive hybridization or differential display.

Discussion and Notes:

The polyA Spin mRNA Isolation Kit complements and serves as an alternative to traditional column chromatography isolation of mRNA. Each polyA Spin spin column has the same column performance as a standard 100 mg column of high quality oligo (dT)-cellulose in the 1.0 mg total RNA sample range. Thus the shorter purification times and reduced working volumes associated with spin chromatography are realized while maintaining column chromatography levels of performance. This is of particular importance in the isolation of low abundance messenger RNA (1). For total RNA samples greater than 1.0 mg initial "first round" isolation of poly(A)$^+$ material should be done by column or "batch" (NEB #S1408S) chromatography (2). First round isolation of poly(A)$^+$ RNA by either column or spin chromatography typically yields a product contaminated with poly(A)$^-$ RNA. PolyA Spin spin chromatography is ideal for "second round" purification, yielding material with an $A_{260/280}$ of 1.9 or greater with minimal product loss. The protocol has been optimized for the isolation of poly(A)$^+$ RNA from 0.1–1.0 mg of total RNA isolated from eukaryotic tissue or cells that contain between 1–5% mRNA. The amount of poly(A)$^+$ RNA isolated will vary with the type of tissue or cells used.

*Note: Please read and become familiar with the manual before starting mRNA isolation.

Protocol:

Isolation of mRNA

Note: Precautions should be taken to avoid ribonuclease contamination during the isolation procedure. Wear latex gloves or equivalent at all times when handling kit components. Any glassware used should be treated prior to its use (3). All kit components have been treated to ensure that they are ribonuclease free.

Preparation:

- **Allow oligo $(dT)_{25}$-cellulose, column(s) and buffers to come to room temperature.**
- **Prepare a 65–70°C bath and an ice bath.**
- **Spin tube containing oligo $(dT)_{25}$-cellulose in a microcentrifuge for 10 seconds. Using a micropipette remove storage buffer. Be careful to avoid drawing of cellulose beads into pipette tip.**
- **Equilibrate cellulose by adding 200 µl of Wash Buffer to cellulose beads, mix thoroughly then microcentrifuge for 10 seconds. Using a micropipette decant supernant.**
- **Prewarm Elution Buffer in 70°C bath.**

Additional Components Required:

- **Ethanol (95%)**
- **Sterile 13 x 100 mm disposable test tubes**
- **Microcentrifuge**
- **Note: It is recommended that microcentrifuge be set at 2,000 to 5,000 x g. Do not exceed 12,000 x g.**
- **Rotary or other type of bidirectional shaker**

Isolation Procedure:

1. Add 50 µl of 5M NaCl per 450 µl of cell lysate or total RNA solution or dissolve total RNA sample in 450 µl of Elution Buffer then add 50 µl of 5M NaCl. Make sure that sample is totally dissolved. If not, microcentrifuge for 5 minutes to pellet insoluble material. Transfer RNA solution to clean microcentrifuge tube.
2. Heat at 65°C for 5 minutes and quickly cool in an ice bath for 3 minutes.

   For information regarding the preparation of total RNA from eukaryotic cells or tissue see Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, second edition, pp. 7.6–7.25 (1989).

3. Apply total RNA solution to equilibrated oligo $(dT)_{25}$ -cellulose, seal cap and mix thoroughly. Let stand at room temperature for 5 minutes agitating by hand or place horizontally on rotary shaker.
4. Microcentrifuge for 10 seconds.

   Note: It is important to agitate beads during binding, washing and elution steps.
5. Pipet supernatant back into original microcentrifuge tube.
   Repeat steps 2 thru 4.
6. Pipet supernatant back into original microcentrifuge tube for storage.

   It is recommended that no spin-column eluates be discarded until entire isolation procedure is completed and the results are evaluated.
   Eluates can be stored in sterile test tubes on ice.
7. Add 400 µl of Wash Buffer to oligo $(dT)_{25}$ -cellulose beads. Agitate by hand to resuspend the cellulose beads. Using a 1 ml micropipette with sterile pipette tip, transfer Wash Buffer and beads to the column reservoir of a clean microcentrifuge spin column unit (provided with kit).
8. Let stand at room temperature for 2 minutes agitating by hand or place horizontally on rotary shaker. Microcentrifuge for 10 seconds. Remove column reservoir and transfer column eluent to a clean 13 x 100 cm test tube.
9. Add 400 µl of Wash Buffer to column reservoir and wash as in Step 8 three times.
10. Using the same method wash column once with 400 µl of Low Salt Buffer.

    *Note: This wash step with 0.1 M NaCl removes residual poly(A)– RNA which is bound to the cellulose column. This step can be omitted during a second round purification.
11. Remove spin-column reservoir and place in a clean microcentrifuge tube (provided with kit).
12. Add 200 µl of prewarmed Elution Buffer to column reservoir. Agitate by hand resuspending the cellulose beads. Let stand for 2 minutes agitating by hand or place on rotary shaker. Microcentrifuge for 10 seconds.
13. Repeat Step 12 using fresh prewarmed Elution Buffer.
14. Place Elution Buffer eluate on ice.

At this point quantification of isolated poly(A)$^+$ can be done by spectrophotometric measurement at A_{260} nm. This is most easily done in a 0.5 ml quartz cuvette.
Soak cuvette for at least one hour in methanol: concentrated HCl (1:1), then rinse copiously with H_2O which has been DEPC-treated.

For RNA 1 A_{260} U = approx. 40 µg

If second round purification is to be done immediately, equilibrate fresh oligo $(dT)_{25}$ -cellulose beads and repeat isolation procedure starting at step 1. If poly(A)$^+$ eluate is to be used at this point it can be ethanol precipitated:

a) To the poly(A)$^+$ eluent add 44 µl of 3 M Sodium Acetate, 20 µl of Glycogen Carrier and 1.0 ml of cold 95% ethanol. Allow to stand at –20°C for at least 30 minutes.

At this point poly(A)$^+$ material can be stored as an ethanol precipitate at –70°C until it is needed.

To recover poly(A)$^+$ material microcentrifuge for 15 minutes at 4°C. Carefully decant supernant, then wash the pellet (often not visible) with 70% ethanol. Recentrifuge briefly, decant supernant and allow pellet to air dry.

Resuspend poly(A)$^+$ material as required.

Troubleshooting:

Problem: Clogged columns.

Suggestions: In cases where a column filter becomes clogged, using a 1 ml micropipette with a sterile tip transfer the cellulose beads and supernant to the microcentrifuge tube of clogged spin column. Continue mRNA isolation using **"batch protocol".** See Appendix. The protocols are nearly identical, differing only at two steps. It is recommended that the elution be done in a clean spin column (there are 2 additional spin columns provided with each kit). This yields a more concentrated final product.

In cases where further isolations from a particular source that has previously clogged a column are to be done it may be advisable to shear sample by passing back and forth through a sterile 18 gauge needle or use a commercially available shearing device. Isolations can also be done using the "batch protocol". This may be done when a total RNA preparation is known to contain large quantities of polysaccharides, genomic DNA or other such debris. Both methods of isolation are equally facile with little difference in yields. When doing batch isolations it is recommended that elutions are done using the spin protocol method.

Problem: Low first -round purity.

Suggestions: Check quality of total RNA preparation. Preparations containing large quantities of polysaccharides or genomic DNA will lower the purity of isolated poly(A)$^+$ RNA. Isolated poly(A)$^+$ RNA will usually contain small amounts of genomic DNA if present in initial sample. Poly(A)$^+$ RNA isolated using polyA Spin is usually 60- 80% pure. This is sufficient to make probes for Northerns, translation experiments, subtractive hybridization or oligo (dT) primed cDNA libraries. Isolated mRNA that will be used to make random primed cDNA libraries should be subjected to a second round of purification.

Genomic DNA Contamination

If genomic DNA is present in total RNA sample it will likely be observed as a contaminant in first round isolated poly(A)$^+$ eluent. A second round of mRNA isolation will serve to remove genomic DNA.

Problem: Low Yield of mRNA.

Suggestions: Enough mRNA is usually isolated for downstream applications. If the total RNA sample is very small (< 100 ug) or desired messenger RNA is of low abundance the low salt wash may be omitted. This may lower mRNA purity but should serve to increase yield.

Appendix:

Batch Method Protocol

Isolation Procedure

1. Add 50 µl of 5M NaCl per 450 µl of cell lysate or total RNA solution or dissolve total RNA sample in 450 µl of Elution Buffer then add 50 µl of 5M NaCl. Make sure that sample is totally dissolved. If not, microcentrifuge for 5 minutes to pellet insoluble material. Transfer RNA solution to clean microcentrifuge tube.
2. Heat at 65°C for 5 minutes and quickly cool in an ice bath for 3 minutes.

   For information regarding the preparation of total RNA from eukaryotic cells or tissue see Maniatis, T. et al. (1989). *Molecular Cloning: A Laboratory Manual*, (2nd ed.) (pp. 7.6–7.25). Cold Spring Harbor.
3. Apply total RNA solution to equilibrated oligo $(dT)_{25}$ cellulose, seal cap and mix thoroughly. Let stand at room temperature for 5 minutes agitating by hand or place horizontally on rotary shaker.
4. Microcentrifuge for 10 seconds.

   Note: It is important to agitate beads during binding, washing and elution steps.
5. Pipet supernant back into original microcentrifuge tube. Repeat steps 2 thru 4.
6. Pipet supernant back into original microcentrifuge tube for storage.

   It is recommended that no eluates be discarded until entire isolation procedure is completed and the results are evaluated. Eluates can be stored in sterile test tubes on ice.
7. Add 400 µl of Wash Buffer to oligo $(dT)_{25}$ beads, let stand at room temperature for 2 minutes agitating by hand or place horizontally on rotary shaker. Microcentrifuge for 10 seconds, then using a micropipette decant supernant to a clean 13 x 100 mm test tube.

8. Add 400 µl of Wash Buffer to cellulose beads and wash as in Step 7 three times.
9. Using the same method wash cellulose beads once with 400 µl of Low Salt Buffer.

   *Note: This wash step with 0.1 M NaCl removes residual poly(A)$^-$ RNA that is bound to the cellulose column. This step can be omitted during a second round purification.
10. Add 200 µl of prewarmed Elution Buffer to cellulose beads. Agitate by hand to resuspend the cellulose beads. Let stand for 2 minutes agitating by hand or place on rotary shaker. Using a 1 ml pipette with sterile tip transfer Elution Buffer and beads to the column reservoir of a clean microcentrifuge spin column unit (provided with kit). Microcentrifuge for 10 seconds.
11. Add 200 µl fresh prewarmed Elution Buffer to column reservoir. Agitate by hand resuspending the cellulose beads. Let stand for 2 minutes agitating by hand or place on rotary shaker. Microcentrifuge for 10 seconds.
12. Place Elution Buffer eluate on ice.

At this point quantification of isolated poly(A)$^+$ can be done by spectrophotometric measurement at A_{260} nm. This is most easily done in a 0.5 ml quartz cuvette. Soak cuvette for at least one hour in methanol: concentrated HCL (1:1), then rinse copiously with H_2O which has been DEPC treated.

For RNA 1 A_{260} U = approx. 40 µg

If second round purification is to be done immediately, equilibrate fresh oligo $(dT)_{25}$ cellulose and repeat isolation procedure starting at step 1 of **Spin Method Protocol** (p. 3). If poly(A)$^+$ eluate is to be used at this point it can be ethanol precipitated:

a) To poly(A)$^+$ eluate add 44 µl of 3 M Sodium acetate, 20 µl of glycogen solution and 1.0 ml of cold 95% ethanol. Allow to stand at –20°C for at least 30 minutes.

At this point poly(A)$^+$ material can be stored as an ethanol precipitate at –70°C until it is needed.

To recover poly(A)$^+$ material microcentrifuge for 15 minutes at 4°C. Carefully decant supernant, then wash the pellet (often not visible) with 70% ethanol. Recentrifuge briefly, decant supernant and allow pellet to air dry. Resuspend poly(A)$^+$ material as required.

References:

1. Gilham, P. T. (1964) *J. Amer. Chem. Soc.*, 86, 4982.
2. Farrell, R. E. (1993) *RNA Methodologies Academic Press Inc.*,104–106.
3. Jacobson, A. (1987) *Methods in Enzymology*, 152, 254–257.
4. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, (2nd ed.) (pp.7.3). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Quality Control:

Each lot of the polyA Spin™ mRNA Isolation Kit is assayed for its ability to isolate mRNA from rat liver total RNA.

Ordering Information

PRODUCT	NEB #	SIZE
polyA Spin™ mRNA Isolation Kit	S1560S	8 isolation

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