

Isolation of mRNA by Affinity Chromatography

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1. Introduction

Messenger RNA (mRNA) comprises approximately 1–5% of total cellular RNA. Although the actual amount depends on the type of cell and its physiological state, at any one time approximately 12,000 genes are being transcribed with approximately 500,000 mRNA molecules present in each mammalian cell.

Eukaryotic mRNAs are heterogeneous in size (ranging from 0.5 kb to over 20 kb) and abundance (from fewer than 15 copies to over 20,000 copies per cell). The presence of a terminal stretch of approximately 200 adenosine residues (the polyA tail) on most eukaryotic mRNAs and its absence in ribosomal and transfer RNAs has important practical consequences, as it allows polyadenylated species (messenger RNAs) to be separated from their nonpolyadenylated counterparts (ribosomal and transfer RNAs, which account for over 90% of total cellular RNA).

High-quality mRNA is needed for a number of molecular biology techniques, including cDNA library construction (1,2). Not surprisingly, numerous mRNA extraction kits are now commercially available. All use the same basic principle, described in **Subheading 2.**, which involves the affinity selection of polyadenylated mRNA using oligodeoxthymidylate (oligo (dT)).

2. Materials

All materials used in this procedure should be sterile and of molecular biology grade. All Tris-containing solutions are prepared using RNase-free water and autoclaved. All other solutions, unless otherwise stated, should be treated directly with diethyl pyrocarbonate (DEPC) and autoclaved. DEPC is an efficient, nonspecific inhibitor of RNase activity. It is, however, a carcinogen and should be handled in a fume hood with extreme care. Hands are a major source of RNase activity. Because of this, gloves should be worn for all procedures.

1. RNase-free water: Add 0.1% DEPC to water. Allow to stand overnight at 37°C and autoclave to destroy residual DEPC activity. All solutions except Tris, which inactivates DEPC, can be treated in the same way.
2. SDS (sodium dodecyl sulphate): SDS is dangerous if inhaled and should be weighed in a fume hood. A 10% stock solution is normally prepared. This solution is unstable if

autoclaved, however any residual RNase activity can be destroyed by heating the solution at 65°C for 2 h.

3. Oligodeoxythymidylate-cellulose (oligo(dT)): Oligo (dT) cellulose is available commercially. Although the binding capacity of oligo(dT) cellulose varies between different suppliers, a general rule is to use 25 mg of oligo(dT) for each 1 mg of total RNA. Suspend oligo (dT) cellulose in loading buffer at a concentration of 5 mg per 1 mL loading buffer. Oligo (dT) is insoluble and should be resuspended by gentle tapping or inversion. Do not put it in a vortex. It can be stored either dry at 4°C or in suspended in loading buffer at -20°C.
4. RNase-free glass wool and Pasteur pipets: Wrap both the glass wool and pipets in aluminium foil and bake at 200°C for 2–4 h to remove any RNase activity.
5. 5 M NaCl: Store at room temperature.
6. 3 M Sodium acetate pH6: Store at room temperature.
7. Absolute alcohol: Store at -20°C.
8. 70% ethanol: Prepare this solution using DEPC-treated water. Store at 4°C.
9. Loading buffer: 0.5 M NaCl in 0.5% SDS, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5 (*see Note 1*). Store at room temperature.
10. Elution buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 7.5. The buffer can be stored at room temperature but should be preheated to 65°C prior to use.
11. Recycling buffer: 0.1 M NaOH, which should be prepared immediately before use and used fresh.

3. Methods

3.1. Preparing an Oligo (dT) Column

Oligo (dT) columns are available commercially or can be prepared by using a 1–3 mL syringe. Preparing your own columns is both easy and cheap.

1. Remove the plunger from the syringe and plug the base with glass wool.
2. Add oligo (dT) cellulose to the syringe using a sterile RNase-free Pasteur pipet. The oligo (dT) cellulose will collect, as a column, above the glass wool. The loading buffer will escape through the glass wool and can be discarded. To ensure that the oligo (dT) cellulose is packed and free from air locks, add 3 vol of loading buffer using a pipette and allow the solution to run through the column. The column is now ready for immediate use and should not be allowed to run dry.

3.2. Isolation of Poly(A+) RNA

1. Resuspend the RNA pellet in loading buffer or, if the buffer is in solution, add 1/10th vol of 5 M NaCl (*see Note 1*).
2. Heat denature RNA and immediately load it onto the column (*see Note 2*) and apply 3 vol of loading buffer.
3. Reapply the eluate to the column (*see Note 3*).
4. Wash with 3 vol of loading buffer (*see Note 4*). Discard eluate.
5. Recover the bound poly(A+) mRNA by adding 3 vol elution buffer. Collect the mRNA in a sterile tube on ice (*see Note 5*).
6. The mRNA is precipitated by adding 1/10th vol of 3 M sodium acetate and 2 vol of ice-cold absolute ethanol. An overnight precipitation at -20°C maximizes the precipitation of RNA.
7. Centrifuge at 15,000g for 15 min to pellet the RNA. Discard the supernatant.
8. Wash the RNA pellet in ice-cold 70% ethanol (*see Note 6*). Centrifuge at 15,000g for 5 min to repellet the RNA which may have been disturbed by washing. Discard the supernatant.

9. Dry the RNA pellet. Once it is dry, resuspend it in DEPC-treated water.
10. Assess the purity and integrity of mRNA (*see* **Note 7** and Chapter 11).

4. Notes

1. SDS can be omitted from the loading buffer as it may precipitate in cold or air-conditioned laboratories and clog the column. Residual SDS may coprecipitate with the RNA and interfere with other procedures such as reverse transcription.
2. RNA can be linearized or denatured by heating to 80–90°C for 5 min, cooling quickly on ice (taking care not to cause precipitation of the loading-buffer components) and immediately applying the solution to the column. This reduces the RNA secondary structure and aids binding of the poly (A⁺)-tailed mRNA to the oligo (dT) column.
3. This step increases the yield of poly(A⁺) RNA.
4. The poly (A⁺) tail anneals to oligo (dT) in the presence of high salt (NaCl) concentrations. The further addition of 3 vol of loading buffer ensures nonpolyadenylated RNA species are washed from the column.
5. The oligo (dT) column can be regenerated by washing with 10 vol of recycling buffer followed by re-equilibration with 3 vol of loading buffer. Oligo (dT) can be stored dry at 4°C or resuspended in loading buffer at –20°C until required.
6. This step removes any contaminating salt that may have coprecipitated with the mRNA.
7. Purity of mRNA is measured by its absorbance at 260 nm. (An absorbance of 1.0 at 260 nm is equivalent to 40 mg mRNA). An additional reading at 280 nm allows the A₂₆₀:A₂₈₀ ratio to be calculated. A ratio of 2.0 should be expected using this protocol. The integrity of the mRNA can be assessed by gel electrophoresis as described in Chapter 29, Formaldehyde Gel Electrophoresis of Total RNA. The mRNA should appear on ethidium bromide-stained gels as a smear ranging from 200 bp to greater than 10 kb with no detectable ribosomal RNA. If small amounts of mRNA are added to the gel, visualization by ethidium bromide may be impossible. To circumvent this problem, set up a Northern blot and hybridize using a labeled oligo(dT) primer. (*See* Chapters 37, 38.) Autoradiography should reveal mRNA fragments ranging in size.

References

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