How to isolate mRNA
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Background: mRNAs (messenger RNAs) comprise only a small percentage of all RNA species in a eukaryotic cell, in Neurospora usually ~ 1-6 % *(Lucas et al., 1977; Sturani et al*., 1979). For some applications like preparation of a cDNA library, target preparation for microarray hybridizations or Northern blot analysis of weakly expressed genes, enriched mRNA preparations are preferable to total RNA. Enrichment of eukaryotic mRNAs derived from nuclear encoded genes is done by virtue of their poly(A) tail which in most cases is 30-200 nt long. mRNA or poly(A)-RNA preparation consists of three steps: (1) hybridization of poly(A)-containing RNAs to oligo-dT molecules connected to a carrier, (2) washing off nucleic acids which do not bind to oligo-dT, (3) elution of poly(A)-RNA from the oligo-dT/carrier combination under low stringency conditions.

Procedure: It is most important for isolation of intact, full length mRNAs to keep an RNase-free environment. All reaction tubes, pipet tips, solutions, etc. used to handle (m)RNA should be RNase-free. Glassware can be baked at 180 °C for 6 h, plastic disposables and solutions should be autoclaved twice. Solutions which cannot be autoclaved should be prepared in sterilized vessels with sterilized water and filter sterilized. Wear gloves throughout.

Poly(A)-RNA preparation can be done using cellulose-bound oligo-dT (Aviv and Leder, 1972; Chirgwin *et al*., 1979), but several other carriers for the oligo-dT molecules have been developed, *e.g*. streptavidin-coupled magnetic beads used in combination with biotinylated oligo-dT or oligo-dT-coupled polystyrene-latex beads. The oligo-dT/carrier combinations are available separately from several manufacturers, but it is more convenient to use a kit which has the advantage of containing most of the necessary reagents pre-packaged in RNase-free quality (*e.g*. polyATtract from Promega or Oligotex mRNA kit from Qiagen work well for Neurospora, Table 1). Depending on the kit size, this allows isolation of poly(A)-RNA from 0.25 to 5 mg of total RNA. For most kits, total RNA used for mRNA preparation should have a concentration of ≥ 2 µg/µl. Total RNA can be prepared with any of the commonly used protocols. One should keep in mind, though, that RNA prepared with a standard phenol/chloroform extraction procedure usually also contains genomic DNA so that the amount of actual RNA in the solution is lower than calculated from OD measurements. Total RNA should be checked by gel electrophoresis and Northern blot for the absence of degradation before using it for mRNA preparation. Visible downward smear from the rRNA bands on a gel or a hybridizing band in a Northern blot indicates degradation of RNA and such preparations should not be used for poly(A)-RNA preparation.

mRNA isolation kits usually incorporate the three steps mentioned above. Here, only a brief outline of the procedure is given (Table 1), details depending on the oligo-dT/carrier combination should be obtained from the manufacturers protocols. First, total RNA is dissolved in a high salt buffer and heated briefly to 65-70 °C to disrupt secondary structures. Afterwards, annealing to oligo-dT is performed at room temperature. The oligo-dT molecules are linked to a carrier which allows washing off non-bound nucleic

acids while retaining the oligo-dT bound poly(A)-RNA. After several washing steps under conditions less stringent than for annealing, poly(A)-RNA is eluted in water or low salt Tris buffer. Elution volumes vary from 20-250 µl for most kits accepting up to 1 mg of total RNA. A yield of 2-30 µg of poly(A)-RNA can be expected from 1 mg of total RNA, but might vary considerably with growth conditions of the mycelium from which the total RNA was extracted. mRNA should be stored in a –80 °C freezer.

Concentration of eluted mRNA can be determined by OD measurement in slightly buffered solution like 10 mM Tris pH 7.0 (OD is pH dependent) using about 1/10 of the eluted volume. Quality of poly(A)-RNA can also be determined by Northern blot hybridization. As a rule of thumb, a transcript which gives a signal in a Northern blot with 20 µg of total RNA should be easily visible as a clear band without any downward smear on 0.1 µg of poly(A)-RNA. To determine size distribution of the eluted mRNAs, such a Northern blot can be probed with oligo-dT which should reveal a smear from ~0.2 to > 3kb. (Amounts less than 1 µg have to be checked by Northern hybridization as they cannot be seen on an agarose gel. To be visible on a gel, at least 1-2 µg poly(A)-RNA have to be used for electrophoresis, and what one usually sees are residual amounts of the ribosomal RNAs.)

For some applications, mRNA has to be concentrated if the elution volume is too big. This can be done by ethanol precipitation, if more than ~800 µg of total RNA were used for mRNA preparation; with less starting material, mRNA amounts may be too low to result in a good recovery. Ethanol precipitation may be done with 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes ethanol at –20 °C overnight.

It should be noted that poly(A)-RNA preparations might still contain some residual genomic DNA. For many applications, this is not a problem; but for any application which involves a PCR step, it might be necessary to perform a DNase treatment before using the mRNA for downstream experiments. Several manufacturers offer DNases and buffer systems which can be used prior to a reverse transcription and PCR step without the need for buffer removal (*e.g.* DNaseI, amplification grade, from Invitrogen).

Table 1: Comparison of two poly(A)-RNA extraction protocols with different oligo-dT/carrier combinations. For details see manufacturers' recommendations.

oligo-dT/carrier combination	biotinylated oligo-dT, streptavidin-coated magnetic beads	oligo-dT bound to polystyrene-latex beads
kit (manufacturer)	polyATtract (Promega)	Oligotex mRNA (Qiagen)
input total RNA	≤ 1 mg[a] in 500 µl water (small-scale),1-5 mg in 2.43 ml water (large-scale)	≤ 0.25 µg in 250 µl water (mini), 0.25-1 mg in 500 µl water (midi), 1-3 mg in 650 µl water (maxi)
binding	incubate 10 min at 65 °C, add oligo-dT and 20x SSC (1x SSC final[b]), let cool to room	add one volume of binding buffer (final concentrations: 10 mM Tris pH 7.5, 0.5 M NaCl, 1 mM EDTA,

	temperature (10-30 min), add to magnetic beads which have been washed with 0.5x SSC three times immediately before use, incubate 10 min at room temperature	0.1 % SDS), incubate 3 min at 70 °C, then 10 min at room temperature
washing	capture magnetic beads to one side of the reaction tube by placing in a magnetic stand (1-2 min), remove supernatant. Resuspend in 0.2x SSC[b],repeatcapturing/resuspending 3x	pellet beads by centrifugation (2 min), remove supernatant. Resuspend in washing buffer (10 mM Tris pH 7.5, 0.15 M NaCl, 1 mM EDTA), centrifuge, remove supernatant, repeat once
elution	resuspend in water, capture particles, collect supernatant containing poly(A)-RNA	resuspend in elution buffer (5 mM Tris pH 7.5) preheated to 70 °C, centrifuge, collect supernatant containing poly(A)-RNA

[a]Use of up to 1.5 mg total RNA from a phenol/chloroform extraction instead of 1 mg as starting material does improve yield.
[b]The original protocol recommends 0.5x SSC for binding and 0.1x SSC for washing, but better results with Neurospora have been obtained with 1x and 0.2x SSC, respectively.

References

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