applied biosystems

Poly(A)Purist™ MAG Kit

USER GUIDE

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Introduction

Overview

Eukaryotic mRNAs contain a stretch of "A" residues at their 3′ ends. The Poly(A)Purist™ MAG Kit uses this characteristic to select mRNA from total RNA preparations. The total RNA used as starting material in this procedure can be prepared from any eukaryotic tissue or cultured cell source using any method, for example Invitrogen™, TRI Reagent™, or RNAqueous™ kits. This kit is optimized to select poly(A) RNA from total RNA in a very low ionic-strength buffer and it cannot be used with crude cell or tissue lysates. The procedure is similar to published methods for Oligo(dT) selection of poly(A) RNA, but the binding and wash solutions are novel. These optimized reagents greatly increase the specificity of poly(A) selection and shorten the procedure. Total RNA in dilute aqueous solution (e.g., water, TE, or THE RNA Storage Solution) is combined with the proprietary Binding Solution, and added to an aliquot of washed Oligo(dT) MagBeads. The mixture is incubated with continual rocking or shaking, allowing hybridization between the poly(A) sequences found on most mRNAs and the Oligo(dT) MagBeads. The supernatant containing unbound material is removed, and the beads are washed to remove nonspecifically bound material and ribosomal RNA. The magnetic property of the Oligo(dT) MagBeads is used throughout the procedure to separate poly(A) RNA (bound to the beads) from total RNA and/or wash solutions. The tube containing the slurry is simply placed on a Magnetic Stand, so that the magnet can draw the Oligo(dT) MagBeads to the side of the tube. Solutions are then removed by gentle aspiration. Finally, the poly(A) RNA is eluted using pre-warmed THE RNA Storage Solution.

The poly(A) RNA can be used immediately after elution from the Oligo(dT) MagBeads, or it can be concentrated by ethanol precipitation. After just a single round of Oligo(dT) selection, the poly(A) RNA will be essentially free of DNA and protein and sufficiently pure for virtually all uses, such as Northern blotting, RT-PCR, microinjection, cDNA library construction, S1 and RNase protection assays, *in vitro* translation, subtractive cDNA cloning and reverse transcription for creating labeled cDNA for gene arrays. The poly(A) RNA can be subjected to a second Oligo(dT) selection to eliminate traces of ribosomal RNA, however, this extra step is rarely required.

Kit components and storage

The Poly(A)Purist™ MAG Kit includes reagents for isolation of poly(A) RNA from 8 mg of total RNA.

Component	Amount	Storage
Nuclease-free Water	5 mL	any temp[1]
Microfuge Tubes	40 each	room temp
Oligo(dT) MagBeads	800 µL	4°C
2X Binding Solution	15 mL	4°C
Wash Solution 1	40 mL	4°C
Wash Solution 2	30 mL	4°C
THE RNA Storage Solution	10 mL	–20°C
5 M Ammonium Acetate	1 mL	–20°C
Glycogen (5 mg/mL)	100 µL	–20°C

[1] Store Nuclease-free Water at –20°C, 4°C, or room temp.

Note: The entire kit is shipped at room temperature which will not affect its stability.

Poly(A)Purist™ MAG Kit procedure

Procedure planning

Starting with total RNA

The Poly(A)Purist™ MAG Kit is optimized for isolation of high quality poly(A) RNA from total RNA in a low ionic-strength environment, and cannot be used with cell or tissue lysates, which routinely contain high levels of interfering ions.

Using the microfuge tubes supplied with the kit

The Poly(A)Purist™ MAG Kit procedure is scalable–in other words, it can accommodate from 30 µg to 8 mg of input total RNA per prep. The type of tube used for the procedure, however, effects the efficiency of rRNA removal. The supplied microfuge tubes give the best results–a microfuge tube can hold a maximum of 1.9 mL, so that 570 µg of total RNA can be processed per tube. 15 and 50 mL tubes have also been extensively tested with the kit–using either tube will yield RNA that contains less rRNA contamination than that recovered using most other commercially available kits. For most molecular biology procedures, such as preparing labeled cDNA for gene array analysis, or doing high-sensitivity Northern blots, a single round of Oligo(dT) selection will yield RNA that is more than sufficiently enriched for poly(A) RNA regardless of what size tube was used. We recommend to either use microfuge tubes or perform 2 rounds of Oligo(dT) selection for applications, such as cDNA library construction, that require the highest level of enrichment for poly(A) RNA.

The volumes of binding and wash solutions needed for the Poly(A)Purist™ MAG Kit procedure will be based on the amount of total RNA used in the prep.

Precipitating RNA to remove salt if necessary

Total RNA prepared from a solid-phase extraction method such as RNAqueous™ can be used immediately after elution because such samples are unlikely to have high levels of salt. On the other hand, RNA isolated by methods that use organic extractions methods (e.g., TRI Reagent™ solution) may have a substantial amount of residual salt. If RNA from these types of procedures has been precipitated only a single time, we recommend doing a second alcohol precipitation to remove residual salt before starting the section, "Prepare total RNA" on page 6).

Saving an aliquot of your total RNA

We recommend that you retain a small aliquot (~1–2 µg if possible) of each total RNA sample to check on a gel after the poly(A) RNA isolation is finished

Prepare total RNA

1. (optional) Alcohol precipitate total RNA to remove residual salt.

   RNA samples, which contain minimal salt left over from the isolation procedure, work best with the Poly(A)Purist™ MAG Kit. If total RNA was isolated using a one-step reagent, or a multi-step organic procedure, and it was precipitated only once as part of the procedure, the RNA should be precipitated again to remove excess salt.

   a. Add the following to the RNA:
      - 0.1 volume 5 M Ammonium Acetate or 3 M sodium acetate

        There may not be enough 5 M Ammonium Acetate supplied with the kit to precipitate large volumes of total RNA. However, the suggested volumes from this procedure are sufficient to produce the amount required (250 µL) for the final precipitation.
      - 1 µL Glycogen

        The glycogen acts as a carrier to increase precipitation efficiency from dilute RNA solutions; it is unnecessary for solutions with ≥200 µg RNA/mL.
      - 2.5 volumes 100% ethanol

   b. Mix thoroughly by vortexing.

   c. Precipitate at –20°C overnight, or quick freeze it in either ethanol and dry ice, or in a –70°C freezer for 30 min.

   d. Recover the RNA by centrifugation at ≥12,000 × *g* for 20–30 min at 4°C.

   e. Carefully remove and discard the supernatant.

      The RNA pellet may not adhere tightly to the walls of the tubes. Therefore, we suggest removing the supernatant carefully, by gentle aspiration with a fine-tipped pipette.

   f. Centrifuge the tube briefly a second time, and aspirate away any additional fluid that collects with a fine-tipped pipette.

   g. Add 1 mL 70% ethanol and vortex the tube a few times.

   h. Repellet the RNA by microcentrifuging, for 10 min at 4°C.

i. Remove supernatant carefully, by gentle aspiration with a fine-tipped pipette.

2. Follow the instructions below for either RNA pellets or for RNA in solution.

   We recommend to start with 30 µg–8 mg total RNA.

   A total RNA concentration of 600 µg/mL is suggested in the following step for optimal poly(A) RNA selection and to most efficiently use the kit reagents. Using a lower concentration will not affect either yield or purity of poly(A) RNA, but it will consume reagents at a higher rate, potentially causing a shortfall of Binding and Wash Solutions. We recommend using a total RNA concentration of less than 1 mg/mL. Using higher concentrations will increase the amount of rRNA left in your prepared sample(s).

   The 30 µg lower limit for input total RNA is the minimum volume that can be handled easily and washed effectively in this procedure. The 8 mg upper limit is imposed by the quantity of reagents supplied with the kit, and is enough for 1 round of Oligo(dT) selection only. As discussed in the "Introduction" on page 4, the Poly(A)Purist™ MAG Kit procedure works slightly better when it is done in microfuge tubes such as those supplied with the kit. If it is inconvenient to use microfuge tubes because the RNA sample is large, and the RNA will be used in an application that requires extremely pure poly(A) RNA, then it may be necessary to do a second round of Oligo(dT) selection.

   Note: For either procedure below, the microfuge tubes supplied with the kit can accommodate up to 570 µg RNA in 950 µL Nuclease-free Water.

   - RNA pellets:
     a. Resuspend 30 µg–8 mg RNA in Nuclease-free Water (included with the kit) to a final concentration of 600 µg/mL.
     b. Vortex vigorously to completely resuspend the pellet.
     c. Add an equal volume 2X Binding Solution and mix thoroughly.
   - RNA in solution:

     It is recommended to start with 30 µg–8 mg RNA in water, TE, or THE RNA Storage Solution.
     a. Add Nuclease-free Water to bring the RNA concentration to ~600 µg/mL.
     b. Add an equal volume 2X Binding Solution to a final volume ≥100 µL.
     c. Mix thoroughly.

Prepare the Oligo(dT) MagBeads

1. Vortex the tube briefly before pipetting to be sure they are well suspended.

   The Oligo(dT) MagBeads are in a 1% (10 mg/mL) suspension.

2. Choose a tube that can accommodate the volume of the total RNA in 1X Binding Solution.

   The microfuge tubes supplied with the kit can be used with up to 1.9 mL of RNA in 1X Binding Solution. For larger volumes, we suggest using RNase-free 15 mL or 50 mL conical tubes with screw-top caps.

3. Put an equivalent mass of Oligo(dT) MagBeads into each tube containing RNA sample.

4. Capture the Oligo(dT) MagBeads by placing the tube on the Magnetic Stand.

   Leave the tube on the stand until all of the Oligo(dT) MagBeads are arranged inside the tube near the magnet. This step will take ~2 min for microfuge tubes, and up to ~10 min for 50 mL conical tubes.

5. Carefully remove the buffer by aspiration and discard it.

   IMPORTANT! To remove solutions from captured Oligo(dT) MagBeads, use gentle aspiration to the bottom of the tube. Be careful not to aspirate any beads that come loose, and avoid using too much force because it can cause the beads to fall away from the magnet.

6. Add Wash Solution 1 to the captured Oligo(dT) MagBeads at a ratio of 500 µL per mg of beads.

7. Remove the tube from the Magnetic Stand and resuspend the beads by inverting the tube several times.

8. Recapture the Oligo(dT) MagBeads with a Magnetic Stand and discard the supernatant.

9. Repeat with a second aliquot of Wash Solution 1.

Bind to Oligo(dT) MagBeads

1. Transfer the total RNA in 1X Binding Solution to the washed Oligo(dT) MagBeads. Seal the tube, and mix by inversion to thoroughly resuspend the suspension.

2. Heat the mixture for 5 min at 65–75°C.

   Incubating the RNA/Oligo(dT) mixture at 65–75°C for 5 min denatures secondary structure and maximizes hybridization between the poly(A) sequences found on most mRNAs, and the poly(T) sequences on the Oligo(dT) MagBeads.

3. Incubate for 30–60 min at room temperature (RT) with gentle agitation.

   Typically 90% of the possible poly(A) binding will occur in first 30 min. If the incubation time is extended to 60 min an additional 5% will occur. Constant rocking or agitation will increase the efficiency of poly(A) RNA binding to the Oligo(dT) MagBeads.

4. Put the container on a Magnetic Stand to pull the Oligo(dT) MagBeads to the side of the tube.

   This step will take longer for larger volumes and more viscous solutions. Of course the strength of the magnet in the stand will also affect the time needed. A rough idea of the time needed for different volumes using our Magnetic Stand is shown below.

Tube size	Approximate time required to capture beads
microfuge (included in kit)	1–2 min
15 mL	5–10 min
50 mL	≤20 min

5. Carefully remove the supernatant by aspiration being careful not to dislodge the Oligo(dT) MagBeads. Save the supernatant on ice until the recovery of poly(A) RNA has been verified.

6. Start preheating the THE RNA Storage Solution to 60–80°C.

   Preheated THE RNA Storage Solution will be used to elute the poly(A) RNA from the Oligo(dT) MagBeads near the end of the procedure.

Wash the Oligo(dT) MagBeads

This wash procedure removes nonspecifically bound material and ribosomal RNA.

1. Add Wash Solution 1 to each tube of Oligo(dT) MagBeads.

   The volume of wash solution should be equal to the initial RNA sample volume (prior to adding 2X Binding Solution).

2. Mix well by inversion or by flicking the tube with a finger.

3. Capture the Oligo(dT) MagBeads on a Magnetic Stand and discard the wash solution.

   IMPORTANT! Remove solutions from captured Oligo(dT) MagBeads by gently aspirating to the bottom of the tube. Be careful not to aspirate any beads that come loose and avoid using too much force because it can cause the beads to fall away from the magnet.

4. Repeat wash for a second time.

   Note: Occasionally, when using nonsiliconized tubes, some beads may adhere to the walls of the tube causing a brown film. This film will not affect either the yield, or the purity of the poly(A) RNA recovered using the kit.

5. Add Wash Solution 2 to each tube of Oligo(dT) MagBeads.

   The volume of wash solution should be equal to the initial RNA sample volume (prior to adding 2X Binding Solution).

6. Mix well by inversion or by flicking the tube with a finger.

7. Capture the Oligo(dT) MagBeads on a Magnetic Stand and discard the supernatant.

8. Repeat wash for a second time.

   IMPORTANT! Be sure to remove as much of this second aliquot of Wash Solution 2 as possible. Also remove any drops of wash solution that may be adhered to the top of the tube.

Recover the poly(A) RNA

Note: In the section, "Wash the Oligo(dT) MagBeads" on page 9, the RNA solution may contain a brown filmy substance—this film will not interfere with subsequent uses of the RNA and can be ignored. Alternatively, it can be removed by centrifuging the tube, and transferring the RNA to a fresh tube

1. Remove the tube from the Magnetic Stand and add 200 µL warm (60–80°C) THE RNA Storage Solution to the Oligo(dT) MagBeads.

   Try to dispense the solution with enough force to suspend the beads. If necessary, close the tube and swirl to suspend the Oligo(dT) MagBeads.

   Note: Most of the poly(A) RNA will be stripped off the Oligo(dT) MagBeads into THE RNA Storage Solution.

2. Capture the Oligo(dT) MagBeads on a Magnetic Stand and transfer THE RNA Storage Solution (containing most of the poly(A) RNA) into a fresh tube.

3. Add a second 200 µL aliquot of prewarmed THE RNA Storage Solution to the Oligo(dT) MagBeads.

4. Resuspend and then capture the Oligo(dT) MagBeads on a Magnetic Stand.

5. Transfer the solution to the tube of poly(A) RNA from step 2. This second elution strips any remaining poly(A) RNA that were bound to the Oligo(dT) MagBeads into THE RNA Storage Solution.

6. Discard the Oligo(dT) MagBeads.

7. Precipitate the eluted poly(A) RNA with the following:

Component	Amount
5 M Ammonium Acetate (NH_4Ac)	0.1 volume
Glycogen[1]	1 µL
100% ethanol	2.5 volumes

[1] The glycogen acts as a carrier, which increases the efficiency of precipitation–it will not interfere with quantitation by UV light absorbance.

8. Either leave the precipitation mixture at –20°C overnight or quick freeze it in either ethanol and dry ice, or in a –70° C freezer for 30 min.

   Note: At this point, the RNA can be stored at –70°C if desired.

9. Recover the RNA by centrifugation at ≥12,000 x *g* for 20–30 min at 4°C.

10. Carefully remove and discard the supernatant.

    The RNA pellet may not adhere tightly to the walls of the tubes. So, we suggest removing the supernatant by gentle aspiration with a fine-tipped pipette.

11. Centrifuge the tube briefly a second time and aspirate any additional fluid that collects with a fine-tipped pipette.

12. (optional) Wash the RNA pellet.
    a. Add 1 mL 70% ethanol and vortex the tube a few times.

b. Repellet the RNA by microcentrifuging for 10 min at 4°C and gently aspirate the supernatent with a fine-tipped pipette.

13. Dissolve the poly(A) RNA pellet in 20–50 µL THE RNA Storage Solution (provided with the kit). If necessary, heat the mixture to 60–80°C to get the RNA into solution.

14. Store the dissolved RNA at –70°C.

(optional) Using microfuge tubes, a second round of Oligo(dT) selection is typically not necessary to obtain poly(A) RNA that is suitable for most molecular biology applications. If the procedure was done in larger tubes, however, and the RNA will be used in an application that requires very pure poly(A) RNA, a second round of Oligo(dT) selection can be performed by repeating the procedure starting from the section, "Prepare the Oligo(dT) MagBeads" on page 7.

Assessing yield and quality of poly(A) RNA

Perform a UV absorbance reading

The concentration and purity of RNA can be determined by diluting an aliquot of the preparation (usually a 1:50 to 1:100 dilution) in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and reading the absorbance in a spectrophotometer at 260 nm and 280 nm. The buffer used for dilution need not be RNase-free (unless you want to recover the RNA), since slight degradation of the RNA will not significantly affect its absorbance.

1. Determine the RNA concentration on a spectrophotometer. (Be sure to zero the spectrophotometer with the TE used for sample dilution.)

   The concentration (µg/mL) of RNA is calculated by multiplying the A_{260} X dilution factor X 40 µg/mL.

   The following example is provided as a reference:

   - An A_{260} of 1 is equivalent to 40 µg RNA/mL.
   - A_{260} = 0.42
   - RNA is resuspended in 40 µL DEPC Water/EDTA
   - 6 µL of the prep is diluted 1:50 into 294 µL of TE

   Therefore, the RNA concentration = 0.42 X 50 X 40 µg/mL = 840 µg/mL or 0.84 µg/µL

2. Calculate the amount of RNA.

   Since there are only 34 µL of the prep left after sacrificing 6 µL to measure the concentration, the total amount of remaining RNA is:

   34 µL X 0.84 µg/µL = 28.56 µg

3. Ascertain the RNA purity.

   The ratio of A_{260} to A_{280} values is a measure of RNA purity, and it should fall in the range of 1.8 to 2.1. Even if an RNA prep has an A_{260}:A_{280} ratio outside of this range, it may function well in common applications such as Northern blotting, RT-PCR, and RNase protection assays.

Perform an ethidium bromide spot assay

1. Make a standard curve with several 2-fold dilutions of an RNA solution of known concentration.

   Using 2 µg/mL ethidium bromide as the diluent, we recommend to start at about 80 ng/µL and end at about 1.25 ng/µL of RNA.

2. Make a few dilutions of the unknown RNA as well.

   The final concentration of ethidium bromide in all the samples should be 1 µg/mL.

3. Spot 2 µL of the RNA standards and the unknown RNA dilutions onto plastic wrap placed on a UV transilluminator.

4. Compare the fluorescence of the RNAs to estimate the concentration of the unknown RNA sample.

5. Make sure that the unknown sample dilutions are in the linear range of ethidium bromide fluorescence.

   This assay will detect as little as 5 ng of RNA with an error of about two-fold.

Perform a fluorescence-based assay

If you have a fluorometer, or a fluorescence microplate reader, our RiboGreen™ RNA Assay Kit is a convenient and sensitive way to measure RNA concentration.

Perform denatured agarose gel electrophoresis

Most poly(A) RNA forms extensive secondary structure via intramolecular base pairing. Because of this, it is best to use a denaturing gel system to size-fractionate RNA. Be sure to include a positive control on the gel so that any unusual results can be attributed to a problem with the gel or a problem with the RNA under analysis. RNA molecular weight markers, an RNA sample known to be intact, or both, can be used for this purpose.

NorthernMax™ reagents for Northern blotting include everything needed for denaturing agarose gel electrophoresis. These products are optimized for ease of use, safety, and low background, and they include detailed instructions for use.

An alternative to using the NorthernMax™ reagents is to use the procedure described below for electrophoresis in a formaldehyde denaturing agarose gel. This procedure is modified from "Current Protocols in Molecular Biology", section 4.9 (Ausubel et al., eds.). It is more difficult and time-consuming than the NorthernMax™ method, but it gives similar results.

1. Dissolve 1 g agarose in 72 mL water and cool to 60°C, for 100 mL of gel solution.

   ⚠ **CAUTION!** Formaldehyde is toxic through skin contact and inhalation of vapors. Manipulations involving formaldehyde should be done in a chemical fume hood.

2. Add 10 mL 10X MOPS running buffer and 18 mL of 37% formaldehyde (12.3 M).

10X MOPS running buffer	
Component	**Concentration**
MOPS, pH 7.0	400 mM
sodium acetate	100 mM
EDTA	10 mM

3. Pour the gel and allow it to set.
   The wells should be large enough to accommodate at least 60 µL.

4. Remove the comb and place the gel in the gel tank.

5. Add enough 1X MOPS running buffer to completely submerge the gel.

6. Plan to run 1 µg of each RNA sample on the gel.

7. Add nuclease-free water to bring the sample volumes to 11 µL.

8. Add the following to each RNA sample.

Component	Amount
10X MOPS running buffer	5 µL
12.3 M formaldehyde	9 µL
formamide	25 µL

9. Heat samples at 55°C for 15 min.

10. Add 10 µL formaldehyde loading dye.

Formaldehyde loading dye	
Component	**Amount**
EDTA	1 mM
bromophenol blue	0.25%
xylene cyanol	0.25%
glycerol	50%
(optional) ethidium bromide	60 µg/mL

11. Load the samples and run the gel at 5 V/cm until the bromophenol blue (the faster-migrating dye) has migrated one-half to two-thirds of the length of the gel.

12. Visualize the gel on a UV transilluminator.
    If ethidium bromide was not added to the formaldehyde loading dye, post-stain the gel for ~20 min in 1X MOPS running buffer with 0.5 µg/mL ethidium bromide and destain with two 10 min incubations in water.

Expected results:

The 28S and 18S ribosomal RNA (rRNA) bands are typically visible in poly(A) RNA–the bands should be sharp and discrete (size is dependent on the organism from which the RNA was obtained). It is difficult to assess the quality of poly(A) RNA from an agarose gel–it should look like a diffuse smear from about 500 bases to about 7 kb, with the majority of the material running at about 2 kb.

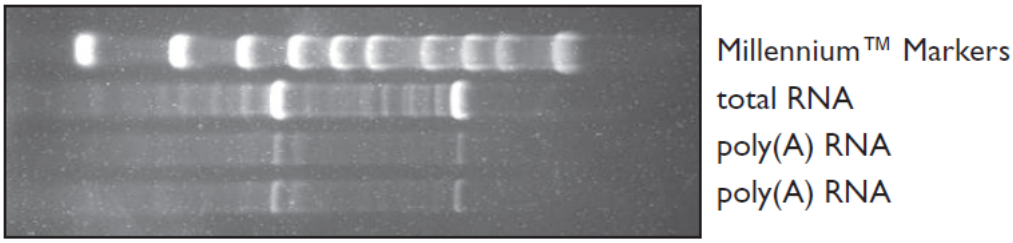

Figure 1 Total RNA and poly(A) RNA

Poly(A) RNA was isolated from 100 µg aliquots of total RNA from mouse liver using the Poly(A)Purist™ MAG Kit. One-quarter of the poly(A) RNA obtained (~0.3 µg), and 1 µg of the input total RNA were fractionated on a 1% agarose denaturing (glyoxal) gel. The samples were pre-stained with ethidium bromide. Note the sharpness of the bands from remaining ribosomal RNA and the background smear of fluorescence from the poly(A) RNA.

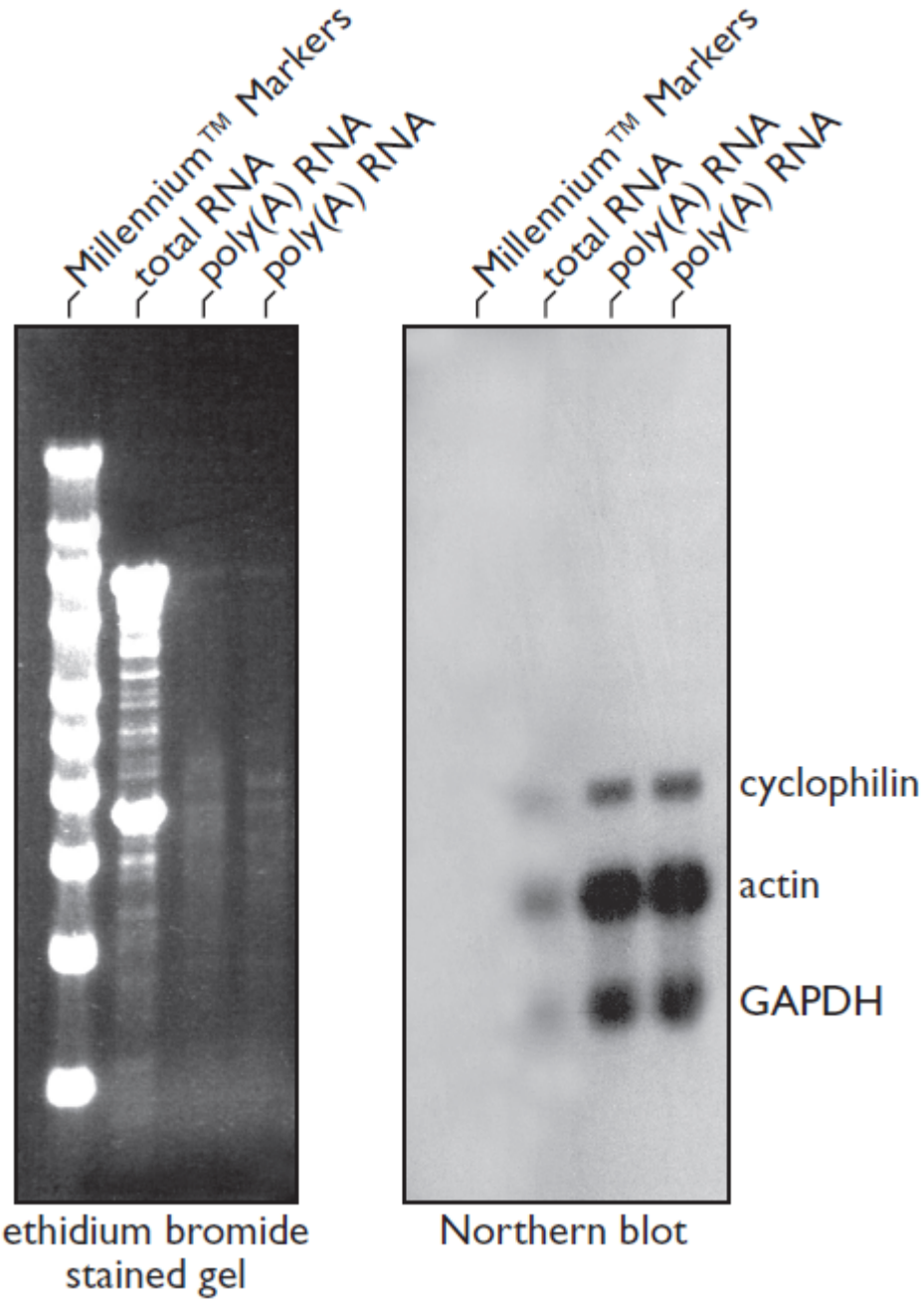

Figure 2 RNA before and after poly(A) selection with the Poly(A)Purist™ MAG Kit

Poly(A) RNA was selected from 80 µg of total RNA from mouse kidney using the Poly(A)Purist™ MAG Kit (single round of Oligo(dT) selection). A 1 µg sample of the RNA was run on an ethidium bromide stained formaldehyde agarose gel next to 2 lanes each containing 25% of the poly(A) RNA recovered from the procedure. The gel was Northern blotted using the NorthernMax™ kit, and was hybridized with probes for the 3 messages shown.

Troubleshooting

Low yield

If the yield of RNA is lower than expected, consider the following explanations and remedies:

- Poly(A) RNA is scarce in the source tissue

  The actual amount of poly(A) RNA depends on cell type and physiological state. Only 1 to 5% of total cellular RNA is poly(A) RNA. Expected yields of poly(A) RNA vary widely among tissues. RNA from liver or kidney tissues are known to have a relatively high proportion of poly(A) RNA in comparison to those from muscle or brain tissue.
- The RNA is degraded

  The total RNA input may have been degraded. Check some of the input total RNA on a denaturing gel (See the section, “Degraded RNA“ on page 16.).

Degraded RNA

- Ruling out gel problems

  If the RNA looks degraded as assessed on a denaturing agarose gel, there could be a problem with the gel, or the RNA could have been exposed to RNase at some point in the procedure. Since high quality poly(A) RNA looks like a smear when run on a gel, degraded poly(A) RNA is difficult to identify by looking at a gel. Therefore, it is important to run an intact control RNA on the gel for comparison.

  If the test RNA looks degraded, but the control RNA produces sharp bands, then the test RNA is probably degraded. There are troubleshooting suggestions on the next few pages for avoiding RNase at each step in the RNA isolation procedure.

  If both the control RNA(s) and the test RNA look smeared, try using fresh reagents for the gel, the running buffer, and the gel loading solution. It is not uncommon for these reagents to go bad after time and use, and this can cause smeary gels.
- Avoiding RNA degradation during sample collection

  To minimize the degradation of poly(A) RNA during sample collection, the tissue should be dissected immediately after sacrificing the source organism, and rapidly extracted or placed in one of the following until it can be extracted:
  - RNA*later*™-ICE Tissue Transition and RNA Stabilization Solution
  - cold phosphate-buffered saline (PBS) on ice
  - liquid nitrogen

  Samples to be stored in RNA*later*™-ICE Tissue Transition and RNA Stabilization Solution can be a maximum of 0.5 cm per dimension. Therefore, many tissue samples must be divided into pieces to allow good penetration of the storage solutions. Smaller pieces freeze faster, and may be easier to manipulate later. Try to remove as much extraneous material as possible from samples that will be frozen or processed fresh. For example, remove adipose tissue from heart, and remove gall bladder from liver. Extraneous material can be removed from tissue stored in RNA*later*™-ICE Tissue Transition and RNA Stabilization Solution at any time. Finally, some tissues benefit from perfusion with cold PBS to eliminate some of the red blood cells.

- Avoiding degradation of RNA during storage

  Instructions for storage of cell and tissue samples in RNA*later*™-ICE Tissue Transition and RNA Stabilization Solutions can be found in the RNA*later*™ Tissue Collection: RNA Stabilization User Guide.

  Cells can be stored in the Lysis Solution at –70°C if desired. They should not be stored as cell pellets because it is difficult to effectively lyse frozen cell pellets.

  Tissue samples can also be snap-frozen by immersion in liquid nitrogen, then transferred to a –70°C freezer for long-term storage. If the pieces are weighed before snap freezing (especially small pieces such as mouse organs), the RNA isolation process will be easier and RNA degradation will be minimized.

  Poly(A) RNA can be damaged by repeated cycles of freeze-thawing (RNA Methodologies, a Laboratory Guide, 1992). To avoid repeated freeze-thawing, poly(A) RNA samples should be stored in small aliquots at –70°C or –80°C in THE RNA Storage Solution provided with the kit.

  If degradation problems are encountered after prolonged storage, it may be desirable to store the RNA as an ethanol precipitate (i.e., add 2 volumes of ethanol to the prep in aqueous solution). The RNA can be recovered by centrifugation, after adjusting the salt concentration to 0.25 M with potassium acetate.

  Alternatively, RNA can be stored in formamide at –20°C–RNase A activity is greatly reduced by storing the RNA in formamide (Chomczynski, 1992).

Impure RNA

- Salt contamination

  Residual salt can inhibit enzymatic reactions, in this procedure, salt can be carried over from the Ammonium Acetate precipitation. Try to avoid this by removing all of the supernatant after the precipitation with the double centrifugation described in the section, “Wash the Oligo(dT) MagBeads“ on page 9. Any remaining salt can be removed by washing the RNA pellet with 70% EtOH as described in step 12 of the "Recover the poly(A) RNA" section on page 10.

- A_{260}:A_{280} ratio below 1.7

  If protein contamination is suspected to be a problem due to a low A_{260}:A_{280} ratio, organic extraction(s) with an equal volume of phenol/CHCl3 or CHCl3/isoamyl alcohol (49:1 or 24:1 mixture) may be beneficial. Chloroform extraction also removes residual phenol. Despite these efforts, the A_{260}:A_{280} ratio may sometimes remain below 1.8, especially for RNA isolated from tissues such as liver and kidney. For most applications, a low A_{260}:A_{280} ratio will probably not affect the results. We have used poly(A) RNA with A_{260}:A_{280} ratios ranging from 1.4 to 1.8 with good results in RNase Protection Assays, Northern blots, *in vitro* translation experiments, and RT-PCR.

- Ribosomal RNA contamination

  Since ribosomal RNA (rRNA) makes up about 80% of total RNA, it is very difficult to recover RNA that does not have some rRNA. Using microfuge tubes for the procedure, a single round of Oligo(dT) selection using Poly(A)Purist™ MAG Kit reduces rRNA to levels acceptable for virtually all molecular biology procedures. When the procedure is done in larger vessels, it may be desirable to add a second round of Oligo(dT) selection for use in procedures that cannot tolerate even trace amounts of ribosomal RNA. To add a second round of Oligo(dT) selection, simply re-start the procedure from the section, “Prepare the Oligo(dT) MagBeads“ on page 7.

A Materials not provided with the kit

Required materials not provided with the kit

- Magnetic stand (e.g. Cat. No. AM10026)
- 100% ethanol (analytical reagent grade)
- Microcentrifuge capable of RCF 12,000 x *g*
- (optional) Materials and equipment for RNA analysis
  - Spectrophotometer
  - Reagents and apparatus for preparation and electrophoresis of agarose gels
  - RiboGreen™ RNA Quantitation Assay and Kit

Related products available from Thermo Fisher Scientific

6 Tube Magnetic Stand (Cat. No. AM10055)	Designed to hold up to six standard microcentrifuge tubes, the 6 Tube Magnetic Stand houses a strong magnet for fast, efficient separation of magnetic beads from solutions.
RNA Isolation Kits	Family of kits for isolation of total or poly(A) RNA. Included in the product line are kits using classical GITC and acidic phenol, one-step disruption/denaturation, phenol-free glass fiber filter or magnetic bead binding, and combination kits.
Electrophoresis Reagents	We also offer gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis.
Millennium™ RNA Markers (Cat. No. AM7150)	Millennium™ RNA Markers are designed to provide very accurate size determination of single-stranded RNA transcripts from 0.5 to 9 kb and can be used in any Northern protocol. They are a mixture of 10 easy-to-remember sizes of *in vitro* transcripts: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 9 kb.

RNase-free Tubes and Tips	RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free.
RNase*Zap*™ Solution (Cat Nos. AM9780, AM9782, AM9784)	RNase*Zap*™ RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*™ Solution.

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C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

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    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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