MicroPoly(A)Purist™ Kit

(Cat #AM1919)

Instruction Manual

Manual 1919M Revision B **Revision Date: September 19, 2007**

For research use only. Not for use in diagnostic procedures.

Literature Citation When describing a procedure for publication using this product, we would appreciate that you refer to it as the MicroPoly(A)Purist™ Kit.

If a paper that cites one of Ambion's products is published in a research journal, the author(s) may receive a free Ambion T-shirt by sending in the completed form at the back of this instruction manual, along with a copy of the paper.

I. Introduction

A. Overview

How it works

Eukaryotic mRNAs contain a stretch of "A" residues at their 3' ends. The MicroPoly(A)Purist Kit (US patent pending) uses this characteristic to select mRNA from total RNA preparations or from small amounts of tissue or mammalian cells. The protocol 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 or small amounts of tissue or cells can be used as starting material

Either total RNA or small amounts of tissue or mammalian cells can be used as starting material with the MicroPoly(A)Purist Kit.

- Start with 2–400 µg total RNA prepared from any eukaryotic tissue or cultured cell source using any method, for example Ambion's TRI Reagent®, RNAqueous®, RiboPure, or TōTALLY RNA™ Kits.
- Start with up to 50 mg animal or plant tissue, or 10^8 mammalian cells

Using total RNA in the MicroPoly(A)Purist procedure offers a robust, rapid method to enrich for poly(A) RNA with less rRNA carryover than any other commercially available system. Also mRNA purity and yield may be slightly better when total RNA is used as the starting material in MicroPoly(A)Purist compared to starting with tissue or cells directly. Skipping total RNA isolation and using tissue or cells directly in MicroPoly(A)Purist is a longer and somewhat more complicated procedure than starting with total RNA, however, it can deliver RNA that is >20 fold enriched for mRNA. When many small samples must be processed, using tissue or cells directly in MicroPoly(A)Purist often saves time over isolating total RNA first.

Procedure overview

(See Figure 1 on page 3.)

Starting from total RNA

Total RNA in dilute aqueous solution (e.g. water, TE, or THE RNA Storage Solution) is combined with the proprietary Binding Solution, and a pre-measured aliquot of Oligo(dT) Cellulose is added to it. The mixture is incubated with continual rocking or shaking, allowing hybridization between the poly(A) sequences found on most mRNAs and the Oligo(dT) Cellulose. The Oligo(dT) Cellulose is then transferred to a Spin Column and washed to remove nonspecifically bound material and ribosomal RNA. Finally, the poly(A) RNA is eluted using pre-warmed THE RNA Storage Solution.

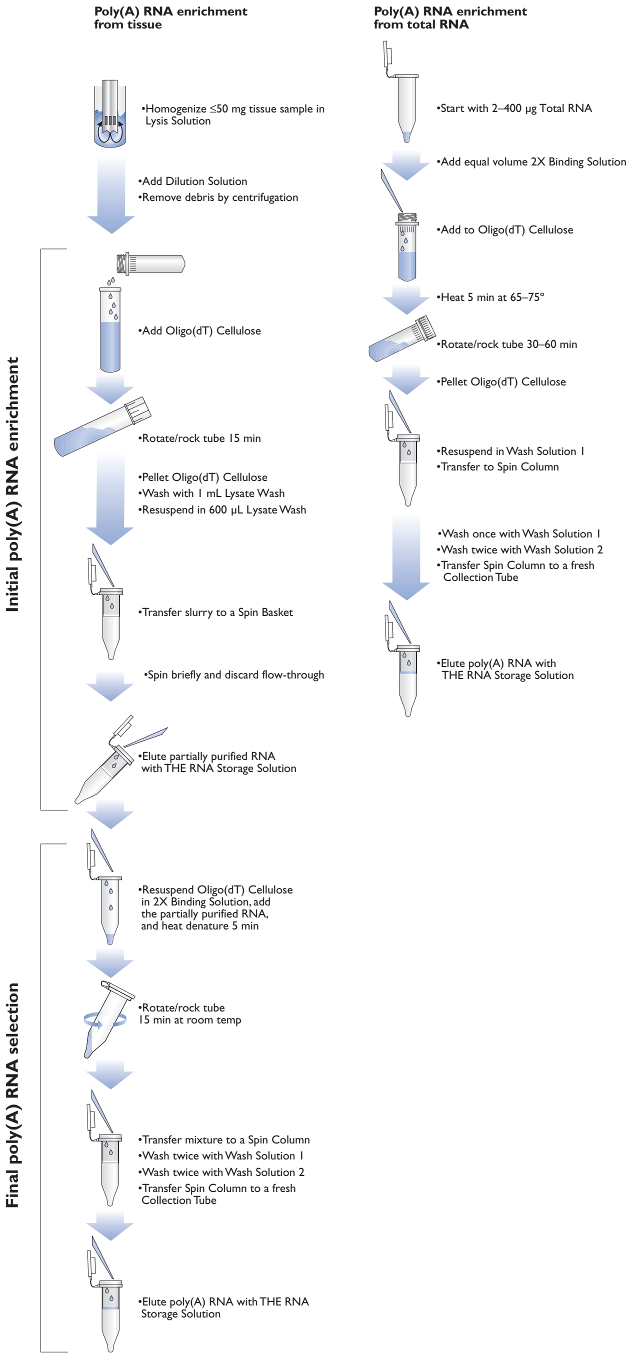
Starting from tissue or cells

When tissue or cells are used as the starting material for mRNA enrichment, the samples are first disrupted and homogenized in a guanidinium-based Lysis Solution. The lysate is diluted and subjected to a preliminary round of poly(A) RNA selection with Oligo(dT) Cellulose. The Oligo(dT) Cellulose is washed and the partially purified RNA is eluted with THE RNA Storage Solution. Then the RNA is subjected to another round of binding and elution with the same batch of Oligo(dT) Cellulose. Typically about 3 µg of highly enriched mRNA can be recovered from 50 mg of tissue or 10^8 mammalian cells.

High quality, highly enriched poly(A) RNA

The poly(A) RNA enriched using the MicroPoly(A)Purist Kit can be used immediately after elution from the Oligo(dT) Cellulose, or it can be concentrated by ethanol precipitation. After completing the procedure [using just a single round of oligo(dT) selection when starting from total RNA], the poly(A) RNA will be essentially free of DNA and protein and sufficiently pure for virtually all uses, such as RT-PCR, Northern blotting, 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 an additional round of oligo(dT) selection to eliminate traces of ribosomal RNA, however, this is rarely required.

Figure 1. MicroPoly(A)Purist Procedure



B. Reagents Provided with the Kit and Storage

The MicroPoly(A)Purist Kit includes reagents for 20 isolations of poly(A) RNA from 2–400 µg of total RNA, from tissue samples up to 50 mg each, or from 10^8 cultured mammalian cells.

Amount	Component	Storage
5 mL	Nuclease-free Water	any temp*
40	Collection Tubes	room temp
20	Collection Tubes w/ Spin Columns	room temp
15 mL	Lysis Solution	4°C
35 mL	Lysate Wash	4°C
40 mL	Dilution Solution	4°C
8 mL	2X Binding Solution	4°C
20 mL	Wash Solution 1	4°C
20 mL	Wash Solution 2	4°C
8 mL	THE RNA Storage Solution	4°C
20 x 20 mg	Oligo(dT) Cellulose	4°C
1 mL	5 M Ammonium Acetate	–20°C
100 µL	Glycogen (5 mg/mL)	–20°C

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

Properly stored kits are guaranteed for 6 months from the date received. Note that the entire kit is shipped at room temperature which will not affect its stability.

C. Required Materials Not Provided with the Kit

- 100% ethanol (analytical reagent grade)
- Microcentrifuge capable of RCF 4,000–12,000 X g
- To start with tissue samples, equipment for tissue disruption is needed: see section III.A starting on page 13 for more information
- ***(optional)*** Materials and equipment for RNA analysis
  - Spectrophotometer
  - Reagents and apparatus for preparation and electrophoresis of agarose gels
  - RiboGreen® RNA Quantitation Assay and Kit (Molecular Probes Inc.)

D. Equipment Preparation

Lab bench and pipettors

Before working with RNA, it is always a good idea to clean the lab bench, and pipettors with an RNase decontamination solution (e.g. Ambion's RNase*Zap*).

Gloves and RNase-free technique

Wear laboratory gloves at all times during this procedure and change them frequently. They will protect you from the reagents, and they will protect the RNA from nucleases that are present on skin.

Use RNase-free pipette tips throughout this procedure, and avoid putting used tips into the kit reagents.

Microfuge tubes

Use the Collection Tubes supplied with the kit; they have been tested for RNase contamination and are certified RNase-free.

Washing/sterilization of equipment

The equipment used for tissue disruption/homogenization should be washed well with detergent and rinsed thoroughly to remove all traces of previous samples. Baking to eliminate RNases is unnecessary, because the Lysis Solution will inactivate any low level RNase contamination.

NOTE

If samples will be ground in a mortar and pestle, pre-chill the equipment in dry ice or liquid nitrogen.

E. Related Products Available from Ambion

RNA Isolation Kits see our web or print catalog	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 binding, and combination organic extraction/glass fiber filter binding kits.
Electrophoresis Reagents see our web or print catalog	Ambion offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis. Please see our catalog or our website (www.ambion.com) for a complete listing as this product line is always growing.
Millennium Markers™ and BrightStar® Biotinylated Millennium Markers™ Cat #7150 and 7170	Ambion's Millennium™ 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 & Tips see our web or print catalog	Ambion's RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com) for specific information.
RNase*Zap*® Cat #9780, 9782, 9784	RNase Decontamination Solution. RNase*Zap* 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*.

II. Poly(A) RNA Isolation from Total RNA

A. Protocol Planning

CAUTION

Spin columns containing Oligo(dT) Cellulose should not be centrifuged at over 5000 X g.

The microfuge tubes supplied with the MicroPoly(A)Purist Kit may not fit in some microcentrifuges, for example some models of microcentrifuges manufactured by Heraeus. We recommend that 1 or 2 tubes be test-spun to make sure that your microcentrifuge rotor is deep enough to accommodate them.

Precipitate RNA to remove salt if necessary

Total RNA prepared from an RNA isolation method that includes a silica filter purification, such as RNAqueous or RiboPure, 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 consisting only of organic extractions, for example using the products TRI Reagent or TōTALLY RNA, 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 MicroPoly(A)Purist procedure (this is described in step B.1 below).

Save an aliquot of your total RNA

It is also a good idea to retain a small aliquot (~1–2 µg if possible) of the total RNA used in this procedure to check on a gel after the poly(A) RNA enrichment is finished.

B. Preparation of Total RNA

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

RNA with minimal salt left over from the isolation procedure will work best in MicroPoly(A)Purist. 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, precipitate the RNA again to remove excess salt.

Add the following to the RNA:

- 0.1 volume 5 M Ammonium Acetate or 3 M sodium acetate

NOTE

There may not be enough 5 M Ammonium Acetate supplied with the kit to precipitate large volumes of total RNA. Using the suggested volumes, ≥250 μL should be reserved for the final precipitation (step E.2 on page 11).

- 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
- Mix thoroughly by vortexing.

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

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

c. 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.

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

e. Add 1 mL 70% ethanol, and vortex the tube a few times. Repellet the RNA by spinning for 10 min at 4°C. Remove supernatant carefully as in steps c and d above.

2. Start with 2–400 μg total RNA (eukaryotic)

Follow the instructions below for either RNA pellets (2a) or for RNA in solution (2b).

2a. RNA pellets: resuspend in water and adjust to 1X Binding Solution in a final volume of 500 μL

- Resuspend 2–400 μg RNA in 250 μL Nuclease-free Water (included with the kit). Vortex vigorously to completely resuspend the pellet.
- Add 250 μL 2X Binding Solution (an equal volume) and mix thoroughly.

2b. RNA solutions: adjust to 1X Binding Solution in a final volume of 500 μL

- Starting with 2–400 μg RNA in water, TE, or THE RNA Storage Solution, add Nuclease-free Water to bring the sample volume to 250 μL.
- Add 250 μL 2X Binding Solution (an equal volume) and mix thoroughly.

C. Bind to Oligo(dT) Cellulose

1. Add each RNA sample to 1 tube Oligo(dT) Cellulose, mix well

Mix by inversion to thoroughly resuspend the resin. If necessary, clumps can be broken up by pipetting up and down.

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

Incubating the RNA/Oligo(dT) Cellulose 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) Cellulose.

3. Rock the tube gently for 30–60 min at room temp

Incubate for 30–60 min at room temp 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 provides maximum efficiency of poly(A) RNA binding to the Oligo(dT) Cellulose.

4. Pellet the Oligo(dT) Cellulose

a. Centrifuge at 4,000 X g for 3 min at room temp.

b. Remove the supernatant by aspiration and save it on ice until the recovery of poly(A) RNA has been verified.

5. Preheat THE RNA Storage Solution to 68–75°C

Preheated THE RNA Storage Solution will be used to elute the poly(A) RNA from the Oligo(dT) Cellulose near the end of the procedure (step E.1 on page 10)

D. Wash the Oligo(dT) Cellulose

1. Wash the Oligo(dT) Cellulose twice with 500 µL Wash Solution 1 each time

These washes remove nonspecifically bound material and ribosomal RNA.

a. Add 500 µL Wash Solution 1 to the Oligo(dT) Cellulose pellet, and vortex briefly to mix well.

b. Place a spin column for each RNA prep into a Collection Tube, and transfer the Oligo(dT) Cellulose suspension to the Spin Column. Check that your microcentrifuge is deep enough for the Collection Tubes before attempting to spin the sample(s).

c. Centrifuge at 4,000 X g for 3 min at room temp to pass the Wash Solution 1 through the Oligo(dT) Cellulose. Discard the flow-through from the Collection Tube, and put the Spin Column back in the tube.

d. Add a second aliquot of 500 µL Wash Solution 1 to the Oligo(dT) Cellulose, close the tube, and vortex briefly to thoroughly mix the wash solution with the cellulose.
Repeat step 1.c (above).

2. Wash the Oligo(dT) Cellulose twice with 500 µL Wash Solution 2 each time

a. Add 500 µL Wash Solution 2 to the Oligo(dT) Cellulose, close the tube over the Spin Column, and vortex briefly to thoroughly mix.

b. Centrifuge at 4,000 X g for 3 min at room temp to pass the Wash Solution 2 through the Oligo(dT) Cellulose. Discard the flow-through from the Collection Tube, and put the Spin Column back in the tube.

c. Repeat steps a and b (above) with a second 500 µL aliquot of Wash Solution 2.

E. Recover the Poly(A) RNA

IMPORTANT

All centrifugations in this section should be at ~5,000 X g at RT.

1. Elute the poly(A) RNA with 200 µL preheated THE RNA Storage Solution

a. Place the Spin Column into a new Collection Tube (provided with the kit).

b. Add 200 µL preheated (68–75°C) THE RNA Storage Solution to the Oligo(dT) Cellulose. Close the tube over the Spin Column and vortex briefly to thoroughly mix.

c. Immediately centrifuge at ~5000 X g for 2 min.
THE RNA Storage Solution strips the poly(A) RNA from the Oligo(dT) Cellulose. The poly(A) RNA is now at the bottom of the microfuge tube.

d. Discard the spin column. [If you intend to do a second round of oligo(dT) selection without first checking the results from a single round, reserve the The Oligo(dT) Cellulose for the second round and repeat the procedure starting at step II.B.2b on page 9.]

2. (optional) Precipitate the RNA

a. Add the following to the eluted poly(A) RNA:

Amount	Component
20 µL	5 M Ammonium Acetate
1 µL	Glycogen*
550 µL	100% ethanol

* The glycogen acts as a carrier which increases the efficiency of precipitation; it will not interfere with quantitation by UV light absorbance.

b. 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.

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

d. 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.

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

3. (optional) Wash the pellet with 70% ethanol

Add 1 mL 70% ethanol, and vortex the tube a few times. Repellet the RNA by spinning for 10 min at 4°C. Remove supernatant carefully as described in steps 2.d and e above.

4. Resuspend the poly(A) RNA in THE RNA Storage Solution

Dissolve the poly(A) RNA pellet in 5–50 µL THE RNA Storage Solution (provided with the kit). If necessary, heat the mixture to 60–80°C to get the RNA into solution.

We recommend storing the RNA at –70°C.

5. (optional) Second round of oligo(dT) selection

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 desired, however, you can add a second round of oligo(dT) selection by simply repeating the protocol starting at step II.B.2b on page 9.

III. Poly(A) RNA Isolation from Tissue or Cells

CAUTION

Spin columns containing Oligo(dT) Cellulose should not be centrifuged at over 5000 X g.

The microfuge tubes supplied with the MicroPoly(A)Purist Kit may not fit in some microcentrifuges, for example some models of microcentrifuges manufactured by Heraeus. We recommend that one or two tubes be test-spun to make sure that your microcentrifuge is deep enough to accommodate them.

A. Sample Disruption and Homogenization

Amount of starting material

This procedure is designed for small scale poly(A) RNA isolation from plant and animal tissue or cells. The following chart lists the amounts of different types of starting material recommended for RNA isolation with a single aliquot of Oligo(dT) Cellulose.

Material	Amount	Instructions
Animal or plant tissue	up to 50 mg*	"Animal or plant tissue samples" (below)
Mammalian cells	up to 1 x 10^8 cells	"Mammalian cultured cells" on page 16

* Larger samples can be used, but they must be split into aliquots derived from ≤50 mg for poly(A) RNA selection.

Animal or plant tissue samples

a. **Collect samples**

For a good yield of intact RNA, it is very important to harvest tissue quickly and to limit the time between obtaining tissue samples and inactivating RNases in step b below.

- Harvest tissue and remove as much extraneous material as possible, for example remove adipose tissue from heart, and remove gall bladder from liver. The tissue can be perfused with cold PBS if desired to eliminate some of the red blood cells.
- If necessary, quickly cut the tissue into pieces small enough for either storage or disruption. Weigh the tissue sample (this can be done later for samples that will be stored in RNA*later*).

b. **Inactive RNases by one of the following methods:**

- Drop the sample into RNA*later*—tissue must be cut to ≤0.5 cm in at least one dimension.

- Disrupt the sample in Lysis Solution. This option is appropriate for fresh tissue samples that are soft to medium consistency, and for small (<0.5 cm^3) frozen tissue samples that are of soft to medium consistency.
- Freeze the sample in liquid nitrogen—tissue pieces must be small enough to freeze in a few seconds. Once frozen, remove tissue from the liquid nitrogen and store it in an airtight container at –80°C.

c. Choose a method for tissue disruption

The method used to disrupt tissue samples depends on the nature of the tissue, the storage method, and the size of the sample; Table 1 (below) shows guidelines for choosing a tissue disruption method.

Table 1. Recommended Tissue Disruption Methods

Sample storage method	Tissue consistency	Suggested disruption method
Any storage method	Very hard	Freeze and grind in liquid N_2 or use a more rigorous method like a bead mill, or a freezer mill
Freshly dissected or stored in RNA*later*	Soft to medium Hard or RNase-rich	Electric or manual homogenizer Freeze and grind in liquid N_2
Frozen	Soft, small pieces (<0.5 cm^3) All other frozen samples	Electric or manual homogenizer Freeze and grind in liquid N_2

NOTE

Comprehensive information on tissue disruption can be found on Ambion's web site (www.Ambion.com—click Technical Resources, then choose The Basics/RNA Isolation, Technical Bulletins 183, 177, or Tips from the Bench/several relevant topics). Technical Bulletins can also be requested through the Ambion Technical Services Department (see the back cover of this booklet for contact info.).

d. Thoroughly homogenize tissue in 12 volumes or at least 200 µL of Lysis Solution

Tissue stored in Ambion's RNA*later*® Solution

Samples in RNA*later* Solution can usually be homogenized by following the instructions for processing fresh tissue. Extremely tough/fibrous tissues in RNA*later* require freezing and pulverization according to the instructions for frozen tissue in order to achieve good cell disruption.

If the samples were immersed in RNA*later* Solution and then frozen, simply thaw samples at room temp before starting. Blot excess RNA*later* from samples, and weigh them before following the instructions for fresh tissue below.

Processing fresh tissue (animal or plant)

i. If the sample weight is unknown, weigh the sample.

ii. Aliquot 12 volumes or at least 200 µL of Lysis Solution into the homogenization vessel.
For example, use 600 µL Lysis Solution for a sample that weighs 50 mg. For very small samples use at least 200 µL of Lysis Solution; this will be >12 volumes.

iii. Mince large samples (≥2 cm^2) rapidly in cold PBS, then remove the PBS before proceeding to the next step. Note that the maximum sample size per RNA isolation in this procedure is 50 mg.

iv. Drop samples into the Lysis Solution, and immediately process to homogeneity. If available, use an electronic rotor-stator homogenizer (e.g. Polytron).

IMPORTANT

Once the tissue is removed from the –70°C freezer, it is important to process it immediately, before even partial thawing can occur. This is necessary because as cells thaw, ice crystals rupture cellular compartments releasing RNase. Partial thawing can cause much of the RNA to be degraded before the RNase is inactivated by the Lysis Solution.

Frozen tissue (animal or plant)

i. If the sample weight is unknown, weigh the sample.

ii. Aliquot 12 volumes or at least 200 µL of Lysis Solution into a plastic weigh boat (we use a plastic weigh boat to simplify transfer of frozen powdered tissue into the Lysis Solution).
For example, if your sample weighs 50 mg, use 600 µL Lysis Solution. For very small samples use at least 200 µL of Lysis Solution; this will be >12 volumes.

iii. Grind frozen tissue to a powder with liquid nitrogen in a pre-chilled mortar and pestle. Alternatively, some frozen tissues can be ground in a coffee grinder with dry ice.

NOTE

With small pieces (<0.5 cm^3) of relatively soft tissue, it is often possible to simply drop the frozen tissue into a vessel containing the Lysis Solution, and homogenize without freezing and grinding in liquid nitrogen.

iv. Using a pre-chilled metal spatula, scrape the powdered tissue into the Lysis Solution (in the weigh boat), then mix rapidly.

v. Transfer the sample to a vessel for homogenization and process the mixture to homogeneity. If available, use an electronic rotor-stator homogenizer (e.g. Polytron).

Once homogenized, lysates can be processed immediately or stored frozen at –80°C for several months.

Mammalian cultured cells

a. **Collect the cells and remove the culture medium**

Suspension cells: pellet the cells at low speed, and discard the culture medium.

Adherent cells: Do one of the following:

i. Aspirate and discard the culture medium.

ii. Trypsinize cells to detach them from the growing surface (use the method employed in your lab for the cell type).

b. **Add 600 µL Lysis Solution for 10^8 cells and lyse the cells. Use a minimum of 200 µL Lysis Solution for <10^8 cells**

In other words, use 200 µL of Lysis Solution for ≤3.3×10^7 cells.

i. Cells will lyse immediately upon exposure to the Lysis Solution.

ii. For adherent cells, collect the lysate with a rubber spatula.

iii. Vortex the cell lysate vigorously or pipette the lysate up and down several times to completely lyse the cells and to obtain a homogenous lysate.

NOTE

Instead of using frozen cell pellets, cells should be lysed as described above if possible, and the lysate should be frozen.

Frozen cell pellets

Grind frozen cell pellets in liquid nitrogen in a mortar and pestle as described for frozen tissue (on page 15). This is necessary because as cells thaw, ice crystals rupture both interior and exterior cellular compartments, releasing RNase.

Very small frozen cell pellets can usually be dropped directly into the Lysis Solution and homogenized immediately without freezing and grinding in liquid nitrogen.

Once homogenized, lysates can be processed immediately or stored frozen at –80°C for several months.

B. Dilute and Clear the Lysate

1. Add 2.33 vol Dilution Solution to the lysate

a. Estimate the lysate volume.

b. Add 2.33 volumes Dilution Solution and mix thoroughly. For example use 1400 µL Dilution Solution for 600 µL of lysate.

2. Centrifuge 15 min at 4°C

Centrifuge at ~12,000 X g for 15 min at 4°C. This will pellet insoluble proteins.

3. Transfer cleared lysate to a fresh tube

Carefully remove the cleared lysate to a fresh RNase-free tube being careful to avoid the pellet of precipitated material.

The tube (not provided with the kit) will be used for the initial Oligo(dT) Cellulose selection, and must have a tight fitting lid (at Ambion, we often use 4 mL snap-cap tubes).

If necessary, split the cleared lysate into several tubes, so that each tube contains cleared lysate made from ≤50 mg of tissue.

C. Initial Poly(A) RNA Enrichment

1. Before starting the procedure:

a. Preheat the THE RNA Storage Solution to 68–75°C.

Preheated THE RNA Storage Solution will be used to elute the partially purified poly(A) RNA from the Oligo(dT) Cellulose in step 7 below.

b. Label the plastic ware.

For each sample label one Spin Column and 3 Collection Tubes with the sample designation.

2. Add 1 tube Oligo(dT) Cellulose to each RNA sample, mix well

a. To each tube containing cleared lysate from ≤50 mg tissue (from step B.3 above), add 1 tube of Oligo(dT) Cellulose.

b. Mix by inversion or vortexing to thoroughly resuspend the resin. If necessary, clumps can be broken up by pipetting up and down.

3. Rock the tube gently for 15 min at room temp

Incubate for 15 min at room temp with gentle agitation. Constant rocking or agitation will provide maximum efficiency of poly(A) RNA binding to the Oligo(dT) Cellulose.

4. Pellet the Oligo(dT) Cellulose

a. Centrifuge at 4,000 X g for 3 min at room temp.

b. Carefully remove the supernatant by aspiration, avoiding the cellulose pellet.

(optional) Save the supernatant containing unbound material on ice until the recovery of poly(A) RNA has been verified.

5. Wash the Oligo(dT) Cellulose with 1 mL Lysate Wash

a. Add 1 mL Lysate Wash to the Oligo(dT) Cellulose pellet, close the tube, and vortex briefly to thoroughly resuspend the Oligo(dT) Cellulose.

b. Centrifuge at 4,000 X g for 3 min at room temp.

c. Carefully remove the supernatant by aspiration avoiding the cellulose pellet and discard.

6. Wash the Oligo(dT) Cellulose with 600 µL Lysate Wash

a. Add 600 µL Lysate Wash to the Oligo(dT) Cellulose pellet, close the tube, and vortex briefly to thoroughly resuspend the Oligo(dT) Cellulose.

b. Place a Spin Column for each RNA prep into a Collection Tube, and transfer the Oligo(dT) Cellulose suspension to the Spin Column. Check that your microcentrifuge is deep enough for the assembled Spin Column/Collection Tubes before attempting to spin the sample(s).

c. Centrifuge at 5,000 X g for 20 sec at room temp to pass the Lysate Wash through the Oligo(dT) Cellulose. Discard the flow-through from the Collection Tube, and reserve it for use in step D.5 on page 19.

7. Elute the partially purified RNA with two 100 µL aliquots of preheated THE RNA Storage Solution

a. Place the Spin Column into a fresh Collection Tube.

b. Pipette 100 µL of THE RNA Storage Solution (pre-warmed to 68–75°C) to the top of the column. Agitate the mixture to create a slurry of Oligo(dT) Cellulose.

c. Centrifuge at 5000 X g for 20 sec.
Most of the poly(A) RNA is now at the bottom of the Collection Tube.

IMPORTANT

Reserve both the Spin Column and the Oligo(dT) Cellulose; they will be reused for the final poly(A) RNA selection.

d. Leave the Spin Column in the tube, and repeat steps b and c above with a second 100 µL aliquot of warm THE RNA Storage Solution. This second elution strips any remaining poly(A) RNA from the Oligo(dT) Cellulose into THE RNA Storage Solution.

D. Final Poly(A) RNA Selection

1. Resuspend the Oligo(dT) Cellulose in 200 µL 2X Binding Solution

Add 200 µL 2X Binding Solution to the reserved Oligo(dT) Cellulose from the initial poly(A) RNA enrichment.

Mix gently stirring the 2X Binding Solution into the cellulose bed, being careful to avoid damaging the filter at the base of the Spin Column.

2. Add the resuspended Oligo(dT) Cellulose to the partially purified RNA

Transfer to resuspended Oligo(dT) Cellulose to the Collection Tube containing the corresponding partially purified RNA from the initial poly(A) RNA enrichment (step C.7.d on page 18). Mix thoroughly.

Reserve the Spin Column for reuse in steps 6–9 below.

3. Heat the mixture for 5 min at 68–75°C

Incubating the RNA/oligo(dT) mixture at 68–75°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) Cellulose.

4. Rock the tube gently for 15 min at room temp

Incubate for 15 min at room temp with gentle agitation. Constant rocking or agitation will provide maximum efficiency of poly(A) RNA binding to the Oligo(dT) Cellulose.

5. Preheat THE RNA Storage Solution to 68–75°C

Preheated THE RNA Storage Solution will be used to elute the poly(A) RNA from the Oligo(dT) Cellulose in step 9.

6. Transfer the Oligo(dT) Cellulose back to the Spin Column, and spin at 5,000 X g for 20 sec

a. Place the reserved Spin Column into the Collection Tube reserved in step C.6.c on page 18. Transfer the Oligo(dT) Cellulose slurry to the assembled Spin Column/Collection Tube.

b. Centrifuge at 5,000 X g for 20 sec at room temp.

c. Remove the flow-through from the Collection Tube. If desired, save the flow-through on ice until the recovery of poly(A) RNA has been verified.

7. Wash the Oligo(dT) Cellulose with two aliquots of 500 µL Wash Solution 1

These washes remove nonspecifically bound material and ribosomal RNA.

a. Add 500 µL Wash Solution 1 to the Oligo(dT) Cellulose, close the cap, and vortex briefly to mix well.

b. Centrifuge at 4,000 X g for 3 min at room temp to pass the Wash Solution 1 through the Oligo(dT) Cellulose. Discard the filtrate from Collection Tube, and put the Spin Column back in the tube.

c. Repeat steps a and b above with a second 500 µL aliquot of Wash Solution 1

8. Wash the Oligo(dT) Cellulose with two aliquots of 500 µL Wash Solution 2

a. Add 500 µL Wash Solution 2 to the Oligo(dT) Cellulose, close the cap, and vortex briefly to mix well.

b. Centrifuge at 4,000 X g for 3 min at room temp to pass the Wash Solution 2 through the Oligo(dT) Cellulose. Discard the flow-through from the Collection Tube, and put the Spin Column back in the tube.

c. Repeat steps a and b above with a second 500 µL aliquot of Wash Solution 2

9. Elute the poly(A) RNA into a fresh Collection Tube with 200 µL preheated THE RNA Storage Solution

a. Place the Spin Column into a fresh Collection Tube (this will be the third Collection Tube used in the procedure).

b. Pipette 200 µL of THE RNA Storage Solution (pre-warmed to 68–75°C) to the top of the column. Agitate the mixture to create a slurry of Oligo(dT) Cellulose.

c. Centrifuge at 5000 x g for 20 sec.
THE RNA Storage Solution strips the poly(A) RNA from the Oligo(dT) Cellulose. The poly(A) RNA is now at the bottom of the Collection Tube.

10. (optional) Precipitate the RNA

The instructions for precipitating the poly(A) RNA are in section II.E, steps 2–4.

IV. Assessing Yield and Quality of Poly(A) RNA

A. Quantitation of RNA

1. UV absorbance

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. Be sure to zero the spectrophotometer with the TE used for sample dilution.

a. Concentration

An A_{260} of 1 is equivalent to 40 µg RNA/mL.

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

b. 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.

2. Fluorescent dye

If you have a fluorometer, or a fluorescence microplate reader, Molecular Probes' RiboGreen® fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration.

3. Ethidium bromide spot assay

Another technique that can be used to quantitate dilute samples of RNA is an ethidium bromide spot assay. 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, start at about 80 ng/µL RNA, and make several 2-fold dilutions, ending about 1.25 ng/µL RNA. 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. Spot 2 µL of the RNA standards and the unknown RNA dilutions onto plastic wrap placed on a UV transilluminator. Compare the fluorescence of the RNAs to estimate the concentration of the unknown RNA sample. 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.

B. Denaturing Agarose Gel Electrophoresis

IMPORTANT

In order to see the ribosomal bands clearly, about 1 μg of RNA must be run on the gel. Depending on the amount of starting material, this may be a significant part of your RNA.

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.

Ambion 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. Prepare the gel

CAUTION

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

a. For 100 mL of gel solution, dissolve 1 g agarose in 72 mL water and cool to 60°C.

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

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

c. Pour the gel and allow it to set. The wells should be large enough to accommodate at least 60 µL. Remove the comb, and place the gel in the gel tank. Cover with a few millimeters of 1X MOPS running buffer.

2. Prepare the RNA samples

a. Plan to run 1 µg of each RNA sample on the gel. Add nuclease-free water to bring the sample volumes to 11 µL.

b. Add the following to each RNA sample

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

c. Heat samples at 55°C for 15 min.

d. Add 10 µL formaldehyde loading dye

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

3. Electrophoresis

a. 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.

b. 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.)

4. 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.

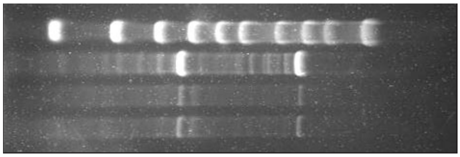


Figure 2. Total RNA and poly(A) RNA

Poly(A) RNA was isolated from 100 µg aliquots of total RNA from mouse liver using the Poly(A)Purist 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.

V. Troubleshooting

A. Low Yield

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

1. 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. If you are accustomed to working with RNA from tissues such as liver or kidney which have a relatively high proportion of poly(A) RNA, you may have unrealistically high expectations of poly(A) RNA yields from tissues such as muscle or brain.

2. The RNA is degraded

The total RNA input may have been degraded. Check some of the input total RNA on a denaturing gel. Also, see below.

B. Degraded RNA

1. Rule 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. That's why 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.

2. Avoiding RNA degradation during sample collection and storage

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:

- Ambion RNA*later*® tissue storage and RNA stabilization solution

- cold phosphate-buffered saline (PBS) on ice
- liquid nitrogen

Samples to be stored in RNA*later* solution can be a maximum of 0.5 cm in one dimension, therefore, many tissue samples must be divided into pieces to allow good penetration of the RNA*later* solution. 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* solution at any time. Finally, some tissues benefit from perfusion with cold PBS to eliminate some of the red blood cells.

Sample storage

Instructions for storage of cell and tissue samples in Ambion's RNA*later* solution can be found in the RNA*later* solution instruction manual.

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. RNA processing will be easier and there will be less opportunity for RNA degradation in the sample if the pieces are weighed before snap freezing (especially small pieces such as mouse organs), to minimize post-freezing manipulation.

3. Avoiding degradation of RNA during storage

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).

C. Impure RNA

1. Residual salt

Salt contamination can inhibit enzymatic reactions, in this protocol, 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 section II.E.2 steps d and e on page 11. Any remaining salt can be removed by washing the RNA pellet with 70% ethanol as described in step 3 on page 11.

2. 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/chloroform or chloroform/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.

3. 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. Typically completing the MicroPoly(A)Purist procedure reduces rRNA to levels acceptable for virtually all molecular biology procedures. To use the RNA in procedures that cannot tolerate even trace amounts of ribosomal RNA, it may be desirable to add another round of oligo(dT) selection. To do this, simply re-start the procedure at step II.B.2b on page 9.

VI. Appendix

A. References

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B. MicroPoly(A)Purist Kit Specification Sheet

Kit contents and storage conditions

Amount	Component	Storage
5 mL	Nuclease-free Water	any temp*
40	Collection Tubes	room temp
20	Collection Tubes w/ Spin Columns	room temp
15 mL	Lysis Solution	4°C
35 mL	Lysate Wash	4°C
40 mL	Dilution Solution	4°C
8 mL	2X Binding Solution	4°C
20 mL	Wash Solution 1	4°C
20 mL	Wash Solution 2	4°C
8 mL	THE RNA Storage Solution	4°C
20 x 20 mg	Oligo(dT) Cellulose	4°C
1 mL	5 M Ammonium Acetate	–20°C
100 µL	Glycogen (5 mg/mL)	–20°C

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

Store kit components at the temperatures specified in the list above. Properly stored kits are guaranteed for 6 months from the date received. Note that the entire kit is shipped at room temperature which will not affect its stability.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

C. Quality Control

Functional testing

All components are tested in a functional RNA Isolation procedure as described in the manual.

Nuclease testing

Kit components are tested in the following nuclease assays:

RNase activity

Meets or exceeds specification when a sample is incubated with ^{32}P-labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity

Meets or exceeds specification when a sample is incubated for 14–16 hr with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity

Meets or exceeds specification when a sample is incubated for 14–16 hr with 40 ng ^{32}P-labeled *Sau*3A fragments of pUC19 and analyzed by PAGE.