The Basics: RNA Isolation

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Obtaining high quality, intact RNA is the first and often the most critical step in performing many fundamental molecular biology experiments, including Northern analysis, nuclease protection assays, RT-PCR, RNA mapping, in vitro translation and cDNA library construction. To be successful, however, the RNA isolation procedure should include some important steps both before and after the actual RNA purification. The following article discusses various RNA isolation procedures and ways of increasing RNA yields.

Tissue or Cell Sample Collection and Disruption

Our ongoing research into optimizing RNA analysis has identified two points in the RNA isolation process that can be improved; treatment and handling of tissue or cells prior to RNA isolation and storage of the isolated RNA. Since most of the actual RNA isolation procedure takes place in a strong denaturant (e.g. GITC, LiCl, SDS, phenol) that renders RNases inactive, it is typically prior to, and after the isolation, when RNA integrity is at risk.

Finding the most appropriate method of cell or tissue disruption for your specific starting material is important for maximizing the yield and quality of your RNA preparation. See the article "**Cell Disruption - Getting the RNA Out,**" which describes various disruption methods and suggests which method to use for specific tissues / cell types, for more information on this subject.

During tissue disruption for RNA isolation, it is crucial that the denaturant be in contact with the cellular contents at the moment that the cells are disrupted. This can be problematic when tissues/cells are hard (e.g. bone, roots), when they contain capsules or walls (e.g. yeast, Gram-positive bacteria) or, when samples are numerous, making rapid processing difficult. A common solution to these problems is to freeze the tissue/cells in liquid nitrogen or on dry ice. The samples must then be ground with a mortar and pestle into a fine powder, which is added to the denaturant. While this freezing and grinding process allows the researcher to postpone RNA isolation, it is a time consuming and laborious process.

Ambion now offers a completely new type of product, a tissue storage buffer that provides more flexibility and time. **RNA*later*™ Tissue Storage:RNA Stabilization Solution** allows the researcher to postpone RNA isolation for days, weeks, or even months after tissue collection without sacrificing the integrity of the RNA. Dissected tissue or collected cells are simply dropped into the RNA*later* solution at room temperature. The solution permeates the cells, stabilizing the RNA. The samples are then stored at 4°C. Samples can be shipped on wet ice or even at room temperature if shipped overnight! Figure 1 shows the integrity of RNA isolated from tissues stored in RNA*later* at 4°C, room temperature and even at 37°C. Samples stored at 4°C

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generate intact RNA even after storage for a month.

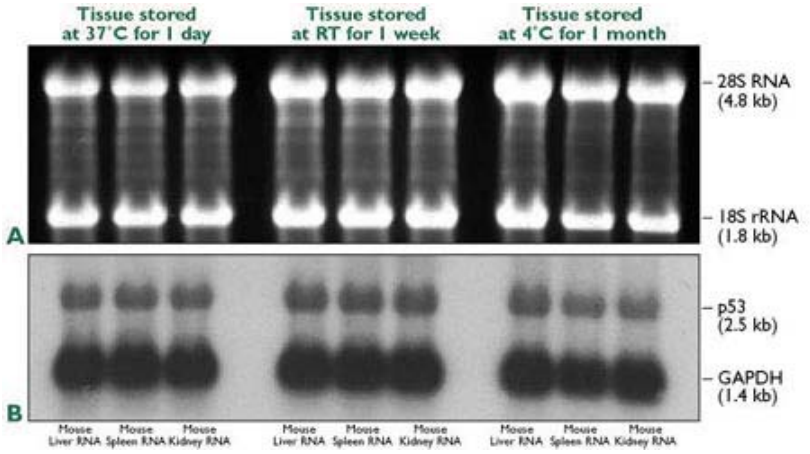

Figure 1. Quality of RNA Isolated from Tissue Stored in RNA*later*™. RNA was purified from the tissues using TRIzol® (Life Technologies). Five µg of each RNA sample was run on a denaturing agarose gel. The top panel shows ethidium bromide-staining of the gel. The bottom panel shows a Northern blot of the same gel hybridized with 10^6 cpm/ml of a high specific activity RNA probe to p53 and 10^4 cpm/ml of a low specific activity RNA probe to GAPDH, using Ambion's **NorthernMax® Kit** reagents and protocol.

Use of RNA*later* for tissue storage is compatible with every RNA isolation procedure that we have tested, including all of Ambion's RNA isolation kits. Tissues stored in RNA*later* are simply removed and processed by homogenization via a dounce, Polytron® (Brinkman), or other mechanical apparatus in the lysis buffer specified by your RNA isolation procedure. See the article "**Cell Disruption - Getting the RNA Out**" for a discussion of sample disruption. Figure 2 shows the RNA isolated from tissue stored in RNA*later* Solution using several methods, and Figure 3 demonstrates that mRNA signal intensities in RPAs are not affected by storage in RNA*later*.

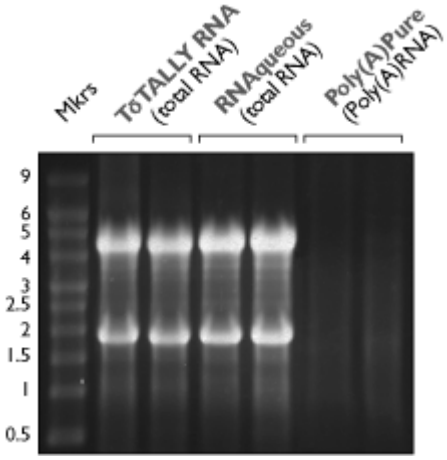

Figure 2. Compatibility of Various RNA Isolation Methods with Tissue Stored in RNA*later*. Freshly dissected whole mouse liver and heart were placed in RNA*later* and stored at 4°C for three days. RNA was isolated from equal mass amounts of each tissue using Ambion's **ToTALLY RNA™**, **RNAqueous®**, or **Poly(A)Pure™** Kits. Five µg of RNA prepared from each tissue sample was analyzed by denaturing formaldehyde agarose electrophoresis. Mkrs - Ambion's **RNA Millennium™ Markers**.

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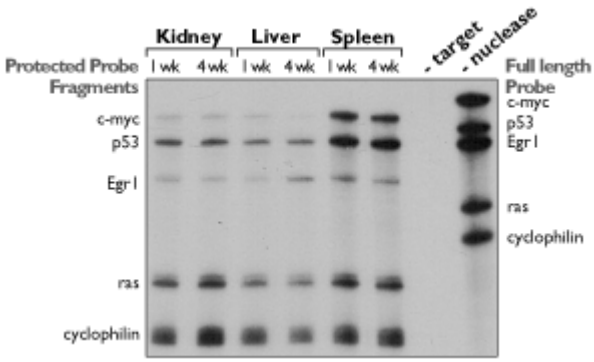

Figure 3. mRNA Profiles of Mouse Tissues Stored in RNA*later*. Various mouse tissues were stored in RNA*later* for 1 or 4 weeks at 4°C. RNA was isolated from each tissue and analyzed using the **RPA III™** Kit. 10 µg of total RNA was hybridized with 5 x 10^4 cpm of each of 5 combined antisense RNA probes, digested with RNase and precipitated. Products were assessed on a 5% polyacrylamide / 8 M urea gel and exposed to film for 4 hours at -80°C with an intensifying screen.

Options for RNA Isolation

Ambion's family of RNA isolation kits provide flexibility for sample size and type, and include kits for the isolation of total or poly(A) RNA. The accompanying table — "**Which RNA Isolation Kit to Choose?**" — summarizes the advantages of each kit to help you determine the optimal RNA Isolation Kit for your particular application. This table also gives information on typical yield per reaction. For additional information on approximately how much total or poly(A) RNA can be recovered from a given amount of tissue or cells, as well as rough expression levels for rare to moderately abundant transcripts, see "**Tissue/Cells to RNA Conversions**."

Total RNA

Eukaryotic Total RNA

Ambion provides several options for isolation of total eukaryotic RNA that are compatible with a variety of cells and tissues, including yeast, plants and animals.

The **RNAwiz™ Reagent** is a single, homogenous solution for the isolation of total RNA. This phenol-based reagent contains a unique combination of denaturants and RNase inhibitors and is used in a convenient, single-step disruption/separation procedure. The tissue or cell sample is homogenized or disrupted in the RNAwiz Reagent, chloroform is mixed with the lysate, and the mixture is separated into three phases by centrifugation. The RNA is then precipitated from the aqueous phase with isopropanol. The entire procedure can be completed in no more than one hour to produce high yields of intact RNA for use in Northerns, nuclease protection assays, RT-PCR and in vitro translation. RNAwiz is especially effective for purifying RNA from microorganisms.

Ambion's **RNAqueous™ Technology** is a rapid, filter-based RNA isolation system that does not require the use of phenol, chloroform or other toxic organic chemicals. The entire procedure can be completed in 20 to 30 minutes, depending on the time required for tissue disruption (see an example of typical results in Figure 4). RNAqueous Technology-based Kits are available in both small and large scale formats. The **RNAqueous Kit** is designed for sample sizes of 10 to 75 mg of tissue or 10^6 to 10^7 cells, whereas the **RNAqueous-Midi Kit** is designed for tissue samples of up to 0.5 g or 10^9 cells. The **RNAqueous-96 Kit** utilizes a 96-well plate format for high-throughput RNA isolation from 100 to 2 x 10^6 cells or 0.1 to 1.5 mg of tissue. The **RNAqueous-4PCR Kit** provides RNA free of genomic DNA contamination from samples as small as 1 mg or 100 cells. The **RNAqueous-Blood Module** can be used in conjunction with the RNAqueous Kit to purify total RNA from blood samples for use in RT-PCR. Ambion's **Plant RNA Isolation Aid** is recommended for use with the RNAqueous Kit for purification of total RNA from plant tissues.

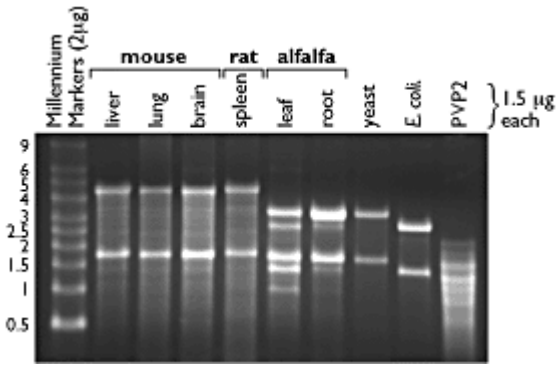

Figure 4. Total RNA from Several Different Tissues and Species Isolated using the RNAqueous® Kit. EtBr-staining of rRNA reveals RNA integrity. The gel is 1% agarose in formaldehyde/MOPS buffer.

Alternatively, total RNA may be isolated using Ambion's **ToTALLY RNA™ Kit**. This procedure is similar to the widely used guanidinium thiocyanate/acid phenol:chloroform method but has been modified to include two formulations of phenol:chloroform and an optional LiCl precipitation step. The modifications help to reduce or eliminate DNA, carbohydrate, heme and other contaminants that can otherwise be difficult to separate from the RNA. Although more time consuming than other RNA isolation procedures — it can take up to 85 minutes from tissue/cell disruption to RNA — reactions can be scaled up or down to accommodate large or small samples.

The **Paraffin Block RNA Isolation Kit** is one of the newest member of Ambion's expanding line of RNA Isolation products, and the first kit of its kind. This kit allows easy isolation of RNA from formalin-fixed, paraffin-embedded tissue sections for use in RT-PCR. The fast, 4-hour protocol yields RT-PCR competent RNA even for rare messages (Figure 5). Paraffin-embedded tissue blocks as old as 16 years have yielded amplifiable RNA.

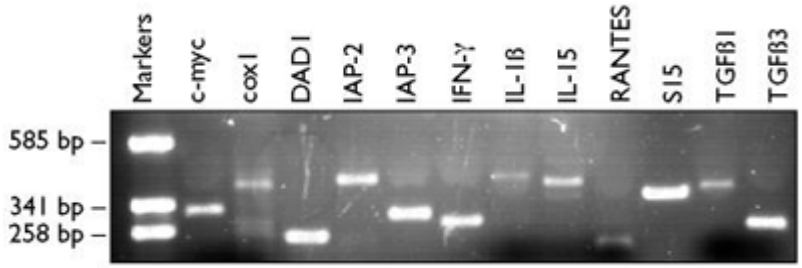

Figure 5. Differentially Expressed Messages Amplified from RNA Isolated using the Paraffin Block RNA Isolation Kit. Total RNA was isolated from two 20 µm mouse heart tissue sections (6-month old block; tissue fixed in 10% neutral buffered formalin for 6 hrs) as per protocol. Ambion's **Gene Specific Relative RT-PCR Kits** were used to amplify twelve distinct genes. Fragments were electrophoresed on a 2% agarose gel.

Prokaryotic Total RNA

Speed is critical in the purification of bacterial RNA due to the short half-life of bacterial mRNA and the need to rapidly "freeze" the mRNA expression profile. Some bacterial isolation protocols call for the pretreatment of bacteria with lytic enzymes (which are usually used in conjunction with a one-step isolation reagent such as **RNAwiz**). While this pretreatment does assist lysis, it delays isolation and may lead to altered expression profiles.

The following methods are better alternatives for effectively freezing gene expression profiles:

- immediate cell lysis and RNA purification
- rapid freezing in liquid nitrogen (a freeze-thaw treatment may help with lysis of some bacteria)

- resuspension of cells in Ambion's **RNA*later***

Easily lysed Gram-negative bacteria may be pipetted directly into a boiling lysis buffer of choice (without even removing the culture medium), and RNA can be immediately extracted with Ambion's **RNAwiz**. Most other bacteria will need to be pelleted by brief centrifugation prior to the above treatments.

To purify RNA from a bacterial cell pellet (including frozen pellets and those stored in RNA*later*), add boiling lysis buffer to the pellet and vortex rapidly, then immediately extract the lysate with RNAwiz or hot acid phenol:chloroform. Harsh mechanical devices (e.g. bead mills) may be required to disrupt some bacterial species. Once lysed, extract the preparation with hot acid phenol:chloroform or RNAwiz. Alternatively, it may be possible to disrupt bacteria directly in Ambion's RNAwiz or acid phenol:chloroform using a bead mill. For more information see "**Purify Bacterial RNA**".

mRNA

Eukaryotic mRNA

Poly(A) RNA (mRNA) makes up between 1-5% of total cellular RNA and is most frequently used for 1) detection and quantitation of extremely rare mRNAs, 2) synthesis of probes for array analysis, and 3) the construction of random-primed cDNA libraries, where the use of total RNA would generate rRNA templates that would significantly dilute out cDNAs of interest. Removal of ribosomal and transfer RNA results in up to a 30-fold enrichment of a specific message. Figure 6 demonstrates the enrichment of a specific message seen after selecting for poly(A) RNA.

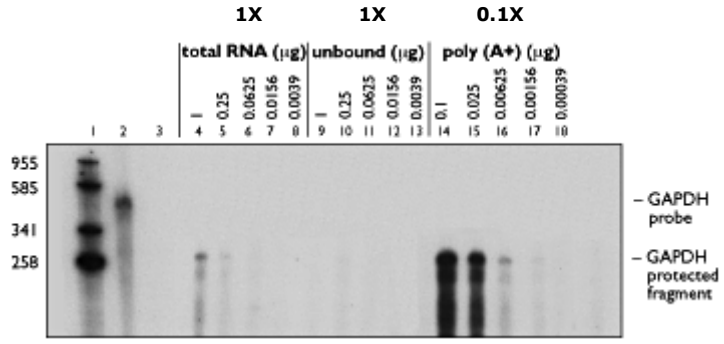

Figure 6. Enrichment of mRNA after Oligo(dT) Selection. The indicated amounts of total RNA from mouse liver, the fraction of RNA that did not bind to oligo dT, and the poly(A)RNA were hybridized with a 300 base antisense RNA transcript for mouse GAPDH. Total yeast RNA was added so that each sample contained 5 µg of RNA. The samples were hybridized and digested with RNase under standard conditions for the **RPA II™ Kit**, leaving only mRNA:probe hybrids. The resulting 250 base protected mRNA:probe duplex was precipitated and run on a 5% polyacrylamide/8 M urea gel and analyzed by autoradiography. Lane 1, **puc19/Sau 3A markers**; lane 2, probe alone control; lane 3, probe + yeast RNA + RNase; lanes 4-8, dilutions of total RNA; lanes 9-13, dilutions of the unbound fraction of total RNA incubated with oligo dT; lanes 14-18, dilutions of poly(A) RNA. (Note: The concentration of poly(A) RNA used was 1/10th the amount examined with total RNA.)

Ambion's **Poly(A)Purist™ Kits** make isolation of mRNA easy by providing a rapid method for isolating the highest possible purity mRNA from total RNA, with the highest possible yield. The Poly(A)Purist and MicroPoly(A)Purist Kits include premeasured aliquots of oligo(dT) cellulose. The Poly(A)Purist MAG Kit utilizes oligo(dT) magnetic bead-based purification. These kits use an optimized hybridization protocol so that mRNA is efficiently bound but without co-isolation of rRNA. These procedures usually require only a single round of oligo(dT) selection to yield mRNA for even the most stringent applications.

For isolation of mRNA directly from tissue or cell lysates, Ambion's original **Poly(A)Pure™ Kits** should be used. Isolation of mRNA direct from tissue or cells saves time and reduces the potential for sample loss and contamination, but the yield and level of purification is somewhat lower than that obtained with the

Poly(A)Purist Kits. Even so, Bioconsumer Review (Spring 1996) ranked Ambion's Poly(A)Pure Kits as having "Highest Yield" and as "Best Direct Kit" in a study that compared many of the mRNA isolation kits currently available. In their article, "Poly(A) Tales," they noted, "The high yields of mRNA obtained, either directly from tissue or from total RNA, speak highly of this kit's value."

Prokaryotic mRNA

For decades mRNA has been isolated from eukaryotic sources using oligo(dT) selection. Bacteria, however, lack the relatively stable poly(A) tails found on eukaryotic messages. Until very recently, isolating mRNA from bacteria has been virtually impossible.

The **MICROB*Express*™ Bacterial mRNA Isolation Kit** employs a novel technology to remove >95% of the 16S and 23S rRNA from total RNA of *E. coli* and other bacterial species The kit is suitable for rapid mRNA purification from a broad spectrum of Gram-positive and Gram-negative bacteria. mRNA isolated with MICROB*Express* is a superior template for synthesizing labeled cDNA for array analysis (Figure 7) and is ideal for quantitative RT-PCR, Northern blotting, and cDNA library construction.

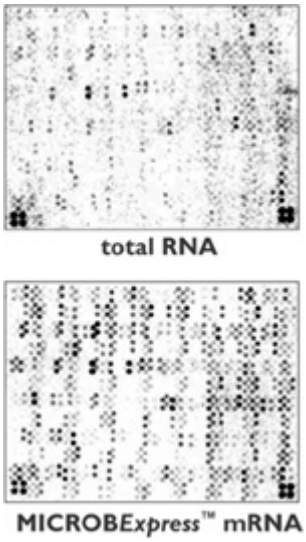

Figure 7. Array Analysis Using *E. coli* mRNA Purified with the MICROB*Express*ª Kit. Replicate Sigma Genosys Panorama[a] *E. coli* Gene Arrays were hybridized with ^{33}P-labeled cDNA synthesized from equal mass amounts (1.5 µg) of either *E. coli* mRNA purifed with MICROB*Express* or *E. coli* total RNA. Random primers were used to prepare labeled cDNA. Arrays were hybridized with equal cpm of labeled cDNA, following the array manufacturer's instructions. There is a dramatic increase in the hybridization signal, but no change in the expression profile when labeled cDNA is prepared from bacterial mRNA instead of from total RNA.

In the first step of the MICROB*Express* procedure, bacterial total RNA is mixed with an optimized set of capture oligonucleotides that bind to the bacterial 16S and 23S rRNAs. Next, the rRNA is removed from the solution using derivatized magnetic microbeads. The mRNA remains in the supernatant and is recovered by ethanol precipitation. The entire procedure takes less than 2 hours.

Storage of Isolated RNA

The last step in every RNA isolation protocol, whether for total or mRNA preparation, is to resuspend the purified RNA pellet. After painstakingly preparing an RNA sample, it is crucial that RNA be suspended and stored in a safe, RNase-free solution. Ambion now has several RNA Storage Solutions for this purpose:

- **THE RNA Storage Solution** (1 mM sodium citrate, pH 6.4 ± 0.2)
- **0.1 mM EDTA** (in DEPC-treated ultrapure water)

- **TE Buffer** (10mM Tris-HCl, 1 mM EDTA, pH 7.0)
- **RNA*secure*™ Resuspension Solution**

Our technical service staff has received numerous requests for pre-made 0.1 mM EDTA and TE Buffers; these solutions are often specified in common RNA isolation and analysis protocols. These storage solutions are ideal for researchers who already use them but would like the convenience and security of having them premade and certified RNase-free.

We are also introducing **THE RNA Storage Solution**, a buffer which delivers greater RNA stability than 0.1 mM EDTA or TE. THE RNA Storage Solution has two features which minimize base hydrolysis of RNA: a low pH, and sodium citrate, which acts both as pH buffer and a chelating agent (divalent cations catalyze base hydrolysis of RNA). THE RNA Storage Solution is compatible with all of the common RNA applications such as reverse transcription, in vitro translation, Northern analysis, and nuclease protection assays.

The **RNA*secure* Reagent** is a unique nonenzymatic reagent for the irreversible inactivation of RNases in enzymatic reactions. RNA*secure*[a] Resuspension Solution contains the same active ingredients as the RNA*secure* Reagent, but is supplied at a working concentration for direct resuspension of RNA pellets. To inactivate RNases, the RNA pellet is resuspended in the RNA*secure* Resuspension Solution and heated to 60°C for 10 minutes. A unique feature of RNA*secure* is that reheating after the initial treatment will reactivate the RNase-destroying agent to eliminate any new contaminants.

Ambion is continuously inventing ways to make RNA analysis easier. Our goal is to provide unique products to solve the problems researchers frequently face when working with RNA. Ambion's RNA*later* Solution, RNA Isolation Kits, and RNA Storage Solutions are designed to work together to take you all the way from tissue to your RNA analysis application. If you have suggestions for additional products that would be useful in your RNA research, please **contact** us.

Ordering Information

Cat#	Product Name	Size
1905	MICROB*Express*™ Bacterial mRNA Enrichment Kit	20 rxns
1910	ToTALLY RNA™ Kit	10 g tissue
1911	RNAqueous®-Midi Kit	15 purifications
1912	RNAqueous® Kit	50 purifications
1914	RNAqueous®-4PCR Kit	30 rxns
1916	Poly(A)Purist™ Kit	6 purifications
1919	MicroPoly(A)Purist Kit	20 purifications
1920	RNAqueous®-96 Kit	192 purifications
1922	Poly(A)Purist™ MAG Kit	Isolation from up to 8 mg total RNA
7000	THE RNA Storage Solution	10 x 1.0 ml
7001	THE RNA Storage Solution	50 ml
7005	RNA*secure*™ Reagent	1 ml
7006	RNA*secure*™ Reagent	10 ml
7010	RNA*secure*™ Resuspension Solution	10 x 1 ml
7020	RNA*later*®	100 ml
7021	RNA*later*®	500 ml
9690	Plant RNA Isolation Aid	10 ml
9736	RNAwiz™	100 ml
9860	TE, pH 7.0	10 x 1.0 ml
9861	TE, pH 7.0	50 ml
9911	0.1 mM EDTA	10 x 1.0 ml

9912	0.1 mM EDTA	50 ml	

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