RNAqueous® Kit

(Part Number AM1912)

Protocol

P/N 1912M Revision C **Revision Date: May 29, 2008**

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I. Introduction

A. Product Description

The RNAqueous® Kit is a rapid, phenol-free, filter based RNA isolation system. It can be used to isolate total RNA from animal and plant tissue, cultured cells, bacteria, yeast, and viral particles. Ambion also offers Plant RNA Isolation Aid (P/N AM9690); a reagent containing a high molecular weight polymer that can be used with the RNAqueous Kit to improve isolation of total RNA from plant tissues.

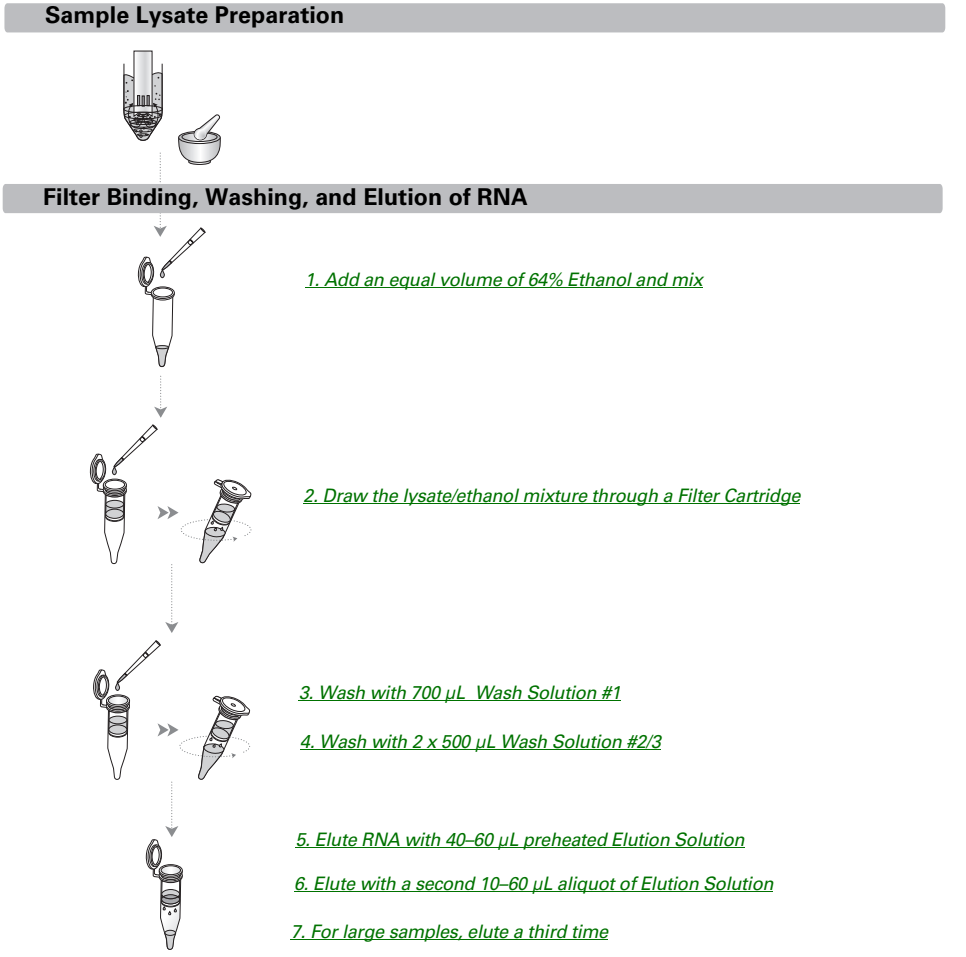
B. Procedure Overview

The RNAqueous method (see Figure 1) is based on the ability of glass fibers to bind nucleic acids in concentrated chaotropic salt solutions (Boom, et al. 1990, Marko, et al. 1982, Vogelstein and Gillespie, 1979). Samples are disrupted in a typical high concentration guanidinium salt solution that simultaneously lyses cells and inactivates endogenous RNases (Chomczynski and Sacchi, 1987). The lysate is diluted with an ethanol solution to make the RNA competent for binding to the glass fiber filter in the RNAqueous Filter Cartridge. This solution is passed through the filter pad where RNA binds and most other cellular contents flow through. The Filter Cartridge is washed 3 times to remove contaminants, and the RNA is eluted in a very low ionic strength solution.

IMPORTANT

*Very low molecular weight RNA is **not** recovered using the RNAqueous kit, thus this procedure is not suitable for isolation of micro RNAs, 5S ribosomal RNAs or tRNA.*

Figure 1. RNAqueous® Kit Procedure



C. Reagents Provided with the Kit and Storage Conditions

This kit contains reagents for 50 RNA isolations.

Note that the kit is shipped at room temperature which will not affect its stability.

Amount	Component	Storage
6 mL	Formaldehyde Load Dye*	–20°C
60 mL	Lysis/Binding Solution†	4°C
60 mL	Water for 64% Ethanol Add 38.4 mL 100% ethanol before use	4°C
4 mL	Lithium Chloride Precipitation Solution	4°C
40 mL	Wash Solution #1†	4°C‡
80 mL	Wash Solution #2/3 Concentrate Add 64 mL 100% ethanol before use	4°C‡
50	Filter Cartridges	room temp
100	Collection Tubes	room temp
10 mL	Elution Solution	any temp**

* This reagent contains formamide and formaldehyde; these are potentially hazardous substances and should be used with appropriate caution.

† These reagents contain guanidinium thiocyanate; this is a potentially hazardous substance and should be used with appropriate caution.

‡ May be stored at room temp for up to 1 month. For longer term storage, store at 4°C, but warm to room temp before use.

**Store Elution Solution at –20°C, 4°C, or room temp.

D. Required Materials Not Provided with the Kit

100% ethanol: ACS grade or higher quality

- For preparation of the 64% Ethanol
- For preparation of Wash Solution #2/3

Apparatus for tissue grinding and homogenization

Liquid nitrogen, mortar and pestle, dry ice are needed for grinding frozen tissue

Tissue homogenizer

- Electronic rotor-stator tissue homogenizers are recommended
- Alternatively, manual homogenizers can be used, we recommend Kontes Duall® ground glass tissue grinders. We have also successfully used smooth-glass manual homogenizers.

Equipment to draw solutions through the RNAqueous Filter Cartridges

With most samples, either a microcentrifuge, or a vacuum manifold apparatus can be used for the RNAqueous procedure.

Microcentrifuge

The microcentrifuge must be capable of attaining an RCF of ≥8000 x g. The centrifugations in this protocol should be done at 10,000–15,000 x g (typically 10,000–14,000 rpm).

Vacuum manifold

- Using a vacuum manifold with an adequately powerful vacuum pump is considerably faster than drawing the solutions through the Filter Cartridges with a microcentrifuge. Relatively weak vacuum pumps will not provide enough force to move lysate through the filter, but they may be adequate for the washing steps.
- Syringe barrels (5 mL) are needed to support the RNAqueous Filter Cartridges on the vacuum manifold.

E. Related Products Available from Applied Biosystems

Electrophoresis Reagents See web or print catalog for P/Ns	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.
High Capacity cDNA Reverse Transcription Kit P/N 4322171, 4368813, 4374967, 4368814, 4374966	The Applied Biosystems High Capacity cDNA Reverse Transcription Kit (formerly the High Capacity cDNA Archive Kit) delivers extremely high-quality, single-stranded cDNA from total RNA. It contains all components necessary for the quantitative conversion of 0.02–2 µg of total RNA to cDNA in a 20 µL reaction.
Millennium™ Markers and BrightStar® Biotinylated Millennium Markers™ P/N AM7150 and AM7170	Ambion 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.
Plant RNA Isolation Aid P/N AM9690	The Plant RNA Isolation Aid contains polyvinylpyrrolidone (PVP) to selectively remove polysaccharides and polyphenolics from plant lysates during RNA isolation. It is compatible with most RNA isolation procedures that use chaotropic salt-based lysis solutions.
RNA*later*® Solution P/N AM7020, AM7021	RNA*later* Solution is an aqueous sample collection solution that stabilizes and protects cellular RNA in intact, unfrozen tissue and cell samples. It eliminates the need to immediately process samples or to freeze samples in liquid nitrogen. Samples can be submerged in RNA*later* for storage at RT, 4°C, or –20°C without jeopardizing the quality or quantity of RNA that can be obtained.
RNA*later*®-ICE Solution P/N AM7030, AM7031	RNA*later*-ICE is a frozen tissue transition solution designed to make it easier to process frozen tissue samples for RNA isolation. Simply drop frozen tissues into RNA*later*-ICE and store overnight at –20°C. Once tissues are treated they can be easily processed using standard RNA isolation procedures.
RNase-free Tubes & Tips See web or print catalog for P/Ns	Ambion 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*® Solution P/N AM9780, AM9782, AM9784	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*.
TaqMan® Universal PCR Master Mix See web or print catalog for P/Ns	Applied Biosystems TaqMan® Universal PCR Master Mix combines the components needed for the fluorogenic 5' nuclease assay in one easy-to-use premix. The proprietary buffer components and stabilizers are optimized to enhance reaction performance across all sample types. TaqMan Universal PCR Master Mix is available with and without uracil-DNA glycosylase, UNG, which prevents carry-over contamination from previous PCRs.
TURBO DNA-*free*™ Kit P/N AM1907	The TURBO DNA-*free* Kit employs Ambion's exclusive TURBO DNase (patent pending); a specifically engineered hyperactive DNase that exhibits up to 350% greater catalytic efficiency than wild type DNase I. The kit also includes a novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation—and without heat inactivation, which can cause RNA degradation. TURBO DNA-*free* is ideal for removing contaminating DNA from RNA preparations.

II. Set-Up and Sample Lysate Preparation

A. Before Using the Kit for the First Time

Prepare 64% Ethanol solution

Add 38.4 mL of 100% ethanol (ACS grade or equivalent) to the bottle labeled Water for 64% Ethanol, which contains 21.6 mL of RNase-free water. Mix well, and mark the empty box on the label to indicate that the ethanol was added.

Prepare Wash Solution #2/3

Add 64 mL of 100% ethanol (ACS grade or equivalent) to the bottle labeled Wash Solution #2/3 Concentrate. Mix well, and mark the empty box on the label to indicate that the ethanol was added.

B. Work Area and Equipment Preparation

Lab bench and pipettors

Before working with RNA, clean the lab bench, and pipettors with an RNase decontamination solution such as Ambion RNase*Zap*® RNase Decontamination Solution.

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 to handle the kit reagents.

Washing/sterilization of equipment

The equipment used for tissue disruption/homogenization should be washed well with detergent and rinsed thoroughly. Baking is unnecessary, because the Lysis/Binding Solution will inactivate any low level RNase contamination.

IMPORTANT

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

C. Amount of Starting Material

This procedure is designed for small scale RNA isolation from plant and animal tissue or cells, bacteria, yeast, viral particles, or enzyme reactions. The limiting factor in determining the maximum amount of starting material in this procedure is clogging of the RNAqueous Filter Cartridge. The following chart lists the amounts of different types of starting material recommended for RNA isolation with a single RNAqueous Filter Cartridge.

Material	Amount
Animal or Plant tissue	1–75 mg
Mammalian Cells	1 x 10^2–1 x 10^7 cells or 1 confluent 60 cm^2 dish
Gram-negative Bacteria	2 x 10^9 cells or 1.5–3 mL culture with an A_{600} ~2–3
Gram-positive Bacteria	10^8–10^9 cells or 1.5–3 mL culture with an A_{600} ~1–2
Yeast	1 x 10^8 cells or 1.5–3 mL culture with an A_{600} ~1–2
Viral Particles	amount expected to contain ~200 µg RNA
Enzyme Reactions	~5–300 µg RNA

Instructions for sample lysis and homogenization of several different sample types are listed below.

D. Animal or Plant Tissue Sample Lysate Preparation

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 3 below.

1. Harvest tissue

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.

2. Cut larger tissue into small pieces

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*® Solution).

3. Inactive RNases by one of the following methods:

- Drop the sample into 5–10 volumes of RNA*later* Solution—tissue must be cut to ≤0.5 cm in at least one dimension. (See the RNA*later* Solution Protocol for detailed instructions.)
- Disrupt the sample in Lysis/Binding Solution (see instructions in step 5 on page 8). This option is only appropriate for fresh tissue samples that are soft to medium consistency.

- Freeze the sample in liquid nitrogen—tissue pieces must be small enough to freeze in a few seconds. When the liquid nitrogen stops churning it indicates that the tissue is completely frozen. Once frozen, remove the tissue from the liquid nitrogen and store it in an airtight container at –80°C.
  Very hard or fibrous tissues (e.g., bone and heart), and tissues with a high RNase content must typically be frozen in liquid nitrogen and ground to a powder for maximum RNA yield.

4. Prepare tissue disruption equipment/supplies

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 tissue disruption methods.

Table 1.Tissue Disruption Methods

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

NOTE

Ambion's website has comprehensive information on tissue disruption. Go to www.ambion.com—click on the document search button and type "disruption" into the search engine.

5. Thoroughly homogenize sample (1–75 mg) in the greater of 200 µL or 10–12 µL/mg of tissue Lysis/Binding Solution

Thoroughly homogenize sample (1–75 mg) in the greater of 200 µL or 10–12 µL/mg of tissue Lysis/Binding Solution following the instructions below for samples stored in RNA*later*®, fresh, or frozen samples.

5a. Preparing samples stored in Ambion RNA*later*® or RNA*later*-ICE Solutions

Samples in RNA*later* Solution can usually be homogenized by following the instructions for fresh tissue in step 5b (next). Extremely tough/fibrous tissues in RNA*later* Solution may need to be frozen and pulverized according to the instructions for frozen tissue (step 5c) in order to achieve good cell disruption.

If the samples were immersed in RNA*later* Solution and then frozen at –80°C, simply thaw samples at room temperature before starting. Blot excess RNA*later* Solution from samples, and weigh them before following the instructions for fresh tissue below.

5b. Fresh animal or plant tissue sample preparation (soft to medium consistency tissues)

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

b. Aliquot the greater of 200 µL or 10–12 µL/mg of tissue Lysis/Binding Solution into the homogenization vessel. For plant samples, you may want to add 1 volume of Plant RNA Isolation Aid. For example, if your sample weighs 50 mg, use 600 µL Lysis/Binding Solution (and 50 µL of Plant RNA Isolation Aid). For very small samples use at least 200 µL of Lysis/Binding Solution; this will be >12 volumes.

c. Mince large samples (≥2 cm^2) rapidly in cold PBS, then remove the PBS before proceeding to the next step (PBS recipe in section VII. on page 26).

d. Drop samples into the Lysis/Binding Solution, and process to homogeneity. If available, use a motorized rotor-stator homogenizer (e.g., Polytron).
Some tissues may need to be frozen in liquid nitrogen and powdered in a mortar and pestle before homogenization to obtain maximum RNA yield and quality.

5c. Frozen, hard-consistency, or RNase-rich tissue sample preparation

After removing the tissue from the freezer, it is important to process it immediately without allowing any thawing. This is necessary because as cells thaw, ice crystals rupture cellular compartments, releasing RNase. By processing samples before they thaw, RNases can be inactivated by the Lysis/Binding Solution before they are released from their cellular compartments.

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

b. Aliquot the greater of 200 µL or 10–12 µL/mg of tissue Lysis/Binding Solution into a a wide-mouth container. (After grinding the tissue in liquid nitrogen, the frozen powder will be transferred to this container—we find that plastic weigh boats work well.) For plant samples, you may want to add 1 volume of Plant RNA Isolation Aid.
For example, if your sample weighs 50 mg, use 600 µL Lysis/Binding Solution and 50 µL of Plant RNA Isolation Aid. For very small samples use at least 200 µL of Lysis/Binding Solution; this will be >12 volumes.

NOTE

Using an electronic rotor-stator homogenizer, small pieces of relatively soft frozen tissues (i.e. <0.5 cm^3) can often be added directly to the Lysis/Binding Solution without first grinding it in a mortar and pestle.

c. Grind frozen tissue to a powder with liquid nitrogen in a pre-chilled mortar and pestle.
Some researchers grind frozen tissue in a coffee grinder with dry ice. Also, sample fragments larger than ~100 mg can be shattered with a hammer.

d. Using a pre-chilled metal spatula, scrape the powdered tissue into the premeasured Lysis/Binding Solution, then mix rapidly.

e. Transfer the slush to a vessel for homogenization and process the mixture to homogeneity. If available, use a motorized rotor-stator homogenizer.

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

E. Mammalian Cultured Cell Lysate Preparation

1. 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

- Aspirate and discard the culture medium from the culture vessel.
- Trypsinize cells to detach them from the growing surface (following the method employed in your lab for the cell type).

2. Add 200–700 µL Lysis/Binding Solution to 100 to 10^7 cells and lyse the cells

a. Add 200–700 µL Lysis/Binding Solution to 100–10^7 cells and vortex or pipette the lysate up and down several times to completely lyse the cells and to obtain a homogenous lysate. Cells will lyse immediately upon exposure to the solution. Use the low end of the volume range (~200 µL) of Lysis/Binding Solution for small numbers of cells (hundreds), and use closer to 700 µL when isolating RNA from larger numbers of cells (millions).

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

c. It is a good idea to shear DNA in lysates from more than ~10^6 cells by sonication or by passing the lysate through a 25 g syringe needle.

3. Frozen cell pellets

IMPORTANT

Instead of using frozen cell pellets, lyse fresh cells as described above if possible, and freeze the lysate.

Grind frozen cell pellets in liquid nitrogen in a mortar and pestle as described for frozen tissue (step D.5b on page 9). This is necessary because as cells thaw, ice crystals rupture both interior and exterior cellular compartments, releasing RNase.

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

F. Bacterial Sample Lysate Preparation

1. Harvest 10^8–10^9 cells by centrifugation. (For many strains, this corresponds to ~3 mL of an overnight culture.) Remove as much of the culture medium as possible.
2. (optional) Enzyme pre-treatment:
   - Resuspend cells in 100 µL TE (10 mM Tris-HCl, 1 mM EDTA) with 1 mg/mL lysozyme or lysostaphin.
   - Incubate 5 min at room temperature for lysozyme, or 15 min at 37°C for lysostaphin to degrade cell envelopes.
3. Add 300 µL Lysis/Binding Solution for up to 3 mL of culture starting volume. (The exact volume of Lysis/Binding Solution is usually not critical and can be adjusted according to user experience.)
4. Vortex vigorously to thoroughly lyse cells.

G. Yeast Sample Lysate Preparation

1. Start with a logarithmically growing culture (A_{600}~1–2); pellet cells and rinse once with nuclease-free water.
   Cells that are grown past the logarithmic phase will usually be more difficult to lyse.
2. Add 300 µL Lysis/Binding Solution per up to 3 mL of culture starting volume.
3. Remove a 2 µL aliquot as a baseline for an absorbance reading at 260 nm. Yeast are difficult to lyse, and an easy way to monitor lysis is by looking for an increase in the A_{260} resulting from the release of nucleic acids.

NOTE

The glass beads will not interfere with RNA isolation, and they can be simply carried along with the lysate for the procedure.

4. Add ~150–200 µL of 0.4–0.5 mm glass beads, and vortex vigorously for 1 min intervals, checking the A_{260} of 2 µL samples after each interval. (Dilute the samples of lysate in 1 mL water to read their absorbance). Lysis will normally be complete after 2–4 rounds of vortexing (1 min each). The A_{260} reading should increase sharply as lysis begins and then level off indicating that lysis is complete.

H. Viral Particle Sample Lysate Preparation

1. Precipitate viral particles using the method appropriate to the organism. Resuspend in 300 µL Lysis/Binding Solution per quantity calculated to contain 200–300 µg RNA.
2. Vortex well to resuspend and solubilize.

I. Enzyme Reactions: Lysate Preparation

RNAqueous can be used to recover RNA from enzyme reactions; it is suitable for the removal of free nucleotides, enzymes, and salts. The recovery from solutions containing less than 5–10 µg of RNA may not be quantitative, therefore, we do not recommend its use with MAXIscript®-type in vitro transcription reactions.

Add 12 volumes Lysis/Binding Solution to the reaction containing ~10–300 µg RNA. Mix thoroughly.

III. RNA Isolation

A. As You Start the Procedure

- Heat an aliquot of Elution Solution (typically ~50–200 µL per prep) in an RNase-free microcentrifuge tube in a heat block set to 70–80°C.
- Briefly inspect the Filter Cartridges before use. Occasionally, the glass fiber filters may become dislodged during shipping. If this is the case, gently push the filter down to the bottom of the cartridge using the wide end of an RNase-free pipette tip.

B. Preparation of Lysate for RNA Isolation

1. Reduce the viscosity of the lysate if necessary

Lysate should be somewhat viscous, but if the solution is very viscous, or contains gelatinous material (which is probably genomic DNA), then it should be sonicated, homogenized in a rotor-stator homogenizer, or passed through a 25 gauge syringe needle several times until the viscosity is reduced. It may be necessary to reduce viscosity by adding more Lysis/Binding Solution and homogenizing with an electronic tissue disrupter.

To continue with the procedure the lysate should be about as viscous as a typical enzyme solution in 50% glycerol.

2. (optional) Centrifuge 2–3 min at top speed in a microcentrifuge to remove debris

This is generally not necessary for samples <25 mg or <10^7 cells.

This centrifugation removes any debris that may be present in the lysate. Most preparations will not have any insoluble material after thorough homogenization.

C. Filter Binding, Washing, and Elution of RNA

CAUTION

Filter Cartridges should not be spun at RCFs over 16,000 x g. Subjecting the filters to centrifugal force higher than this may cause mechanical damage, and/or may deposit glass filter fibers in the final RNA eluate.

1. Add an equal volume of 64% Ethanol and mix

Add an equal volume of 64% Ethanol to the lysate and mix gently but thoroughly by carefully pipetting or vortexing, or by inverting the tube several times.

2. Draw the lysate/ethanol mixture through a Filter Cartridge

a. Apply the lysate/ethanol mixture (from the previous step) to a Filter Cartridge assembled in either a Collection Tube (supplied) or a 5 mL syringe barrel on a vacuum manifold.
The maximum volume that can be applied at one time is ~700 µL.

If you are using a vacuum manifold, apply the vacuum to draw the lysate through the filter. As soon as there is no more liquid resting on top of the filter, the remaining lysate can be applied. Once the entire sample has been passed through the filter, proceed to step 3. Do not release and reapply the vacuum between wash steps, simply leave it on until all of the wash steps are finished.

If the filter clogs, try inserting it into one of the Collection Tubes and centrifuging at RCF 10,000–15,000 x g until the mixture is through the filter.

b. Centrifuge at RCF 10,000–15,000 x g (typically 10,000–14,000 rpm) for ~15 sec–1 min or until the lysate/ethanol mixture is through the filter.

c. Discard the flow-through and reuse the Collection Tube for the washing steps.

d. Repeat as necessary with ~700 µL aliquots until all of the sample has been drawn though the filter. Generally up to ~2 mL of sample mixture can be passed through the filter without clogging or exceeding its RNA binding capacity.

3. Wash with 700 µL Wash Solution #1

Apply 700 µL Wash Solution #1 to the Filter Cartridge.

Draw the washes through the filter as in the previous step. Discard the flow-through and reuse the tube for subsequent washes.

4. Wash with 2 x 500 µL Wash Solution #2/3

a. Add 500 µL Wash Solution #2/3. Draw the wash solution through the filter as in the previous step.

b. Repeat with a second 500 µL aliquot of Wash Solution #2/3.

c. After discarding the wash solution, continue centrifugation, or leave on the vacuum manifold for ~10–30 seconds to remove the last traces of wash solution.

5. Elute RNA with 40–60 µL preheated Elution Solution

a. Put the Filter Cartridge into a fresh Collection Tube.

b. Pipet Elution Solution preheated to ~70–80°C to the center of the filter. Close the cap of the tube.
The exact volume of Elution Solution used is not critical. The amount of Elution Solution should correlate to the amount of RNA expected, in other words, RNA from samples close to the maximum size should be eluted with more Elution Solution than RNA from smaller samples. For maximum elution efficiency it is important to elute the RNA using 2 sequential applications of Elution Solution. The minimum practical volume of Elution Solution to use is 50 µL, applied as sequential aliquots of 40 µL and 10 µL.

c. Recover eluate by centrifugation for ~30 seconds at room temperature (RCF 10,000–15,000 x g).

6. Elute with a second 10–60 µL aliquot of Elution Solution

Add a second aliquot of hot Elution Solution to the center of the filter and re-spin for ~30 seconds.
Typically, this second elution is collected into the same tube as the first elution.

7. For large samples, elute a third time

To recover all of the RNA from samples near the maximum recommended size (i.e. 75 mg of tissue or 10^7 cells), include a third elution by repeating step 5b (above) and eluting into the same tube.

For small samples, including a third elution is not a good idea because the amount of RNA is typically negligible, and it may be contaminated with DNA.


NOTE

Glass fibers can be removed from eluted RNA preps by spinning briefly to pellet the fibers and transferring the RNA to a fresh tube.

D. (optional) Precipitate with LiCl


IMPORTANT

The concentration of RNA should be at least 0.2 µg/µL to assure efficient precipitation. Also, LiCl precipitation will not quantitatively precipitate small RNAs such as tRNA and 5S ribosomal RNA.

Lithium chloride precipitation removes carbohydrates and gross DNA contamination.

1. Mix the RNA well with one-half volume LiCl Precipitation Solution.
2. Incubate at –20°C for at least 30 min.
3. Microcentrifuge 15 min at top speed.
4. Carefully remove and discard the supernatant.
5. Wash pellet with cold 70% ethanol, re-centrifuge, aspirate away the supernatant.
6. Air dry the pellet.

IV. Assessing RNA Yield and Quality

A. Assessing RNA Yield and Purity

RNA yield

Spectrophotometry

The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm (A_{260}) using a spectrophotometer. With a traditional spectrophotometer, dilute an aliquot of the RNA 1:50–1:100 in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and read the absorbance. (Be sure to zero the spectrophotometer with the TE used for sample dilution.) The buffer used for dilution need not be RNase-free, since slight degradation of the RNA will not significantly affect its absorbance.

NanoDrop spectrophotometers are more convenient—no dilutions or cuvettes are needed, just measure 1.5 µL of the RNA sample directly.

To determine the RNA concentration in µg/mL, multiply the A_{260} by the dilution factor and the extinction coefficient (1 A_{260} = 40 µg RNA/mL).

A_{260} x dilution factor x 40 = µg RNA/mL

Be aware that any contaminating DNA in the RNA prep will lead to an overestimation of yield, since all nucleic acids absorb at 260 nm.

Fluorometry

If a fluorometer or a fluorescence microplate reader is available, Molecular Probes' RiboGreen® fluorescence-based assay for RNA quantitation is a convenient and sensitive way to measure RNA concentration. Follow the manufacturer's instructions for using RiboGreen.

RNA quality

Microfluidic analysis

Microfluidic systems such as the Agilent® 2100 bioanalyzer with Caliper's RNA LabChip® Kits provide better quantitative data than conventional gel analysis for characterizing RNA. When used with Ambion RNA 6000 Ladder (P/N AM7152), this system can provide a fast and accurate size distribution profile of RNA samples. Follow the manufacturer's instructions for performing the assay.

The 28S to 18S rRNA ratio is often used as an indicator of RNA integrity. Total RNA isolated from fresh and frozen mammalian tissues using this kit usually has a 28S to 18S rRNA ratio of >1.2.

Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. A metric developed by Agilent, the RIN analyzes information from both rRNA bands, as well as information contained outside the rRNA peaks (potential degradation products) to provide a fuller picture of RNA degradation states. Search for "RIN" at Agilent's website for information:

www.chem.agilent.com

Spectrophotometry

An effective measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm; it should fall in the range of 1.8 to 2.1. Even if an RNA prep has a ratio outside of this range, it may function well in common applications such as RT-PCR, Northern blotting, and RNase protection assays.

B. Denaturing Agarose Gel Electrophoresis

The overall quality of an RNA preparation may be assessed by electrophoresis on a denaturing agarose gel; this will also give some information about RNA yield. A denaturing gel system is suggested because most RNA forms extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to its size. Be sure to include a positive control RNA on the gel so that 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. This denaturing agarose gel method for RNA electrophoresis is modified from "Current Protocols in Molecular Biology", Section 4.9 (Ausubel et al., eds.). It is more 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 that involve formaldehyde should be done in a chemical fume hood.

a. Heat 1 g agarose in 72 mL water until dissolved, then cool to 60°C.

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

Table 2. 10X MOPS Running Buffer

Concentration	Component
0.4 M	MOPS, pH 7.0
0.1 M	sodium acetate
0.01 M	EDTA

c. Pour the gel using a comb that will form wells large enough to accommodate at least 25 µL.

d. Assemble the gel in the tank, and add enough 1X MOPS running buffer to cover the gel by a few millimeters. Then remove the comb.

2. Prepare the RNA sample

a. To 1–3 µg RNA, add 0.5–3 volumes Formaldehyde Load Dye (included in kit).

- To simply check the RNA on a denaturing gel, as little as 0.5 volumes Formaldehyde Load Dye can be used, but to completely denaturate the RNA, e.g. for Northern blots, use 3 volumes of Formaldehyde Load Dye.
- Ethidium bromide can be added to the Formaldehyde Load Dye at a final concentration of 10 µg/mL.
  Some size markers may require significantly more than 10 µg/mL ethidium bromide for visualization. To accurately size your RNA, however, it is important to use the same amount of ethidium bromide in all the samples (including the size marker) because ethidium bromide concentration affects RNA migration in agarose gels.

b. Heat denature samples at 65–70°C for 5–15 min.
Denaturation for 5 min is typically sufficient for simply assessing RNA on a gel, but a 15 min denaturation is recommended when running RNA for a Northern blot. The longer incubation may be necessary to completely denature the RNA.

3. Electrophoresis

Load the gel and electrophorese at 5–6 V/cm until the bromophenol blue (the faster-migrating dye) has migrated at least 2–3 cm into the gel, or as far as 2/3 the length of the gel.

4. Results

Visualize the gel on a UV transilluminator. (If ethidium bromide was not added to the Formaldehyde Load Dye, the gel will have to be post-stained and destained.)

Figure 2 shows a typical denaturing agarose gel containing various RNAs isolated with the RNAqueous Kit. The 28S and 18S ribosomal RNA bands should be fairly sharp, intense bands (size is dependent on the organism from which the RNA was obtained). The intensity of the upper band should be about twice that of the lower band. Smaller, more diffuse bands representing low molecular weight RNAs (tRNA and 5S ribosomal RNA) may be present, however these RNAs are not quantitatively recovered using this kit. It is normal to see a diffuse smear of ethidium bromide staining material migrating between the 18S and 28S ribosomal bands, probably comprised of mRNA and other heterogeneous RNA species. DNA contamination of the RNA preparation (if

present) will be evident as a high molecular weight smear or band migrating above the 28S ribosomal RNA band. Degradation of the RNA will be reflected by smearing of ribosomal RNA bands.

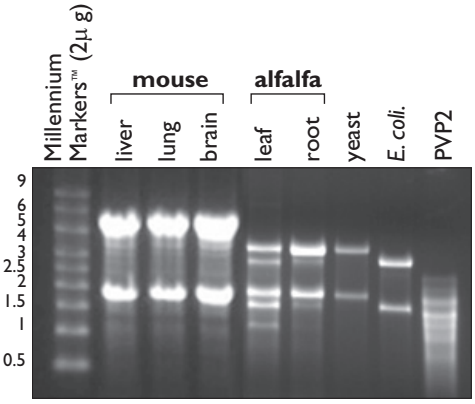


Figure 2. Total RNA (1.5 µg) From Several Different Tissues and Species Isolated Using RNAqueous.

The gel is 1% agarose in formaldehyde/MOPS buffer. Samples were heated at 65°C for 15 min in Formaldehyde Load Dye containing 10 µg/mL ethidium bromide before electrophoresis at 5 V/cm.

V. Troubleshooting

A. Filter Clogging

Lysate is too viscous

Reduce viscosity with one of the following methods:

- Process the lysate with a Polytron-type tissue disrupter.
- Pass the lysate through a syringe needle several times.
- Sonicate the lysate until viscosity is reduced.
- Homogenize the lysate more thoroughly.
- Dilute the lysate with more Lysis/Binding Solution mixed 1:1 with 64% Ethanol.

Use a microcentrifuge if you have been using a vacuum manifold, or centrifuge for a longer time to force the lysate through the filter.

Lysate contains debris

Homogenize more thoroughly.

Remove debris by centrifugation, and use only the supernatant. Any centrifugation of lysate to remove debris must be done ***before*** adding the 64% Ethanol (step 1 on page 13).

B. Problems During Denaturing Gel Electrophoresis

Problems with gel electrophoresis can cause RNA to appear to be degraded. Consider these suggestions if you suspect that electrophoresis was not optimal.

IMPORTANT

Gel problems can be ruled out by running an aliquot of "positive control" RNA, i.e. an archived, intact RNA sample, on the same gel as the RNA preparation being evaluated for the first time.

Ribosomal RNA (rRNA) is overloaded

Running more than about 5 µg of RNA in a single lane may cause smiling and/or smearing of the rRNA bands. rRNA comprises about 80% of total RNA, so if 5 µg of total RNA is loaded in a gel lane, there will be about 1 µg and 3 µg of RNA in the 18S and 28S rRNA bands respectively.

Samples are incompletely denatured

To ***completely*** denature RNA, the sample should be diluted with at least 3 volumes of Formaldehyde Load Dye and then incubated in a 65°C water bath for at least 15 min. A 65°C cabinet type incubator works well, but somewhat longer incubation times may be required due to the lower heat transfer capacity of air. After incubation, transfer the samples immediately to an ice bath. Samples are stable on ice for at least 20 min,

or long enough to add ethidium bromide to the samples if desired and load them on the gel. If an interruption occurs, the samples may be incubated at 65°C again without ill effects.

Gel was run too fast

Smearing may occur if gels are run at more than 5–6 volts/cm as measured between the electrodes. For example, if the distance between the electrode wires in the electrophoresis chamber measures 15 cm, the gel should be run at a constant 75 volts.

Electrophoresis buffer was depleted

For long runs (>3 hr) the buffer may be circulated to avoid the formation of pH gradients in the gel. This can be accomplished in various ways: manual circulation of the buffer every 15–30 min throughout the run (be sure samples have migrated into the gel first), continuous circulation of the buffer from one chamber to the other with a pump, or continuous circulation of the buffer using magnetic stir bars placed in both chambers.

Gel or gel apparatus was contaminated with RNase

RNase contamination of the gel running equipment, reagents, or supplies can cause RNA degradation while the gel is running. To decontaminate equipment, we recommend using Ambion RNA*Zap*® RNase Decontamination Solution following the instructions provided.

C. RNA Degradation

Improper handling of tissue

It is extremely important to inactive RNases as quickly as possible after sample collection to avoid RNA degradation. When samples are obtained from sacrificed animals or cadavers, it is also important to limit the time between death and sample collection for the best yield of high quality RNA.

Frozen tissue thawed before immersion in Lysis/Binding Solution

It is essential that frozen tissue stays frozen until it is disrupted in Lysis/Binding Solution.

If the tissue is frozen in small pieces (i.e. <0.5 cm^3), and it will be processed with an electronic rotor-stator homogenizer (Polytron type), it can often be dropped directly in a vessel containing Lysis/Binding Solution and processed before it has a chance to thaw or to freeze the Lysis/Binding Solution. This shortcut generally only works for relatively soft tissues.

When powdering tissue in a mortar and pestle, it is important to pre-chill the mortar and pestle, and to keep adding small amounts of liquid nitrogen during grinding so that the tissue never thaws, even partially. Once the tissue is completely powdered, it should be mixed with

the Lysis/Binding Solution quickly before any of the powder can thaw. It may be convenient to scrape the frozen powder into a plastic weigh boat containing the volumes of Lysis/Binding Solution.

Exogenous RNase contamination

Once the lysate is bound to the RNAqueous filter matrix, and the Lysis/Binding Solution is removed by the washing steps; all the typical precautions against RNase contamination should be observed. Gloves should be worn at all times, and changed frequently to avoid the introduction of "finger RNases". The bags containing the Collection Tubes, and the solution bottles should be kept closed when they are not in use to avoid contamination with dust. Any tubes or solutions not supplied with the kit, which will contact the RNA, should be bought or prepared so that they are free from RNases.

See Ambion Technical Bulletins #159 and 180 (available on our website www.ambion.com) for more information on avoiding RNase contamination.

Verifying kit component performance

Isolate RNA from a source where tissue handling and lysis are straightforward.

Overnight *E. coli* cultures, or mammalian cells grown in culture, can be lysed simply by vigorous vortexing of the pelleted cells in the Lysis/Binding solution. The ribosomal RNA band should look intact on a denaturing agarose gel. See Figure 2 on page 19 for an example of how minimally degraded RNA should appear on a denaturing agarose gel.

D. RNA Yield is Lower than Expected or Inconsistent

Poor tissue disruption

Often, the best way to disrupt tissue is by grinding frozen tissues in liquid nitrogen, and then homogenizing using a rotor-stator-type homogenizer. It is useful with very tough tissues to break the frozen tissue with a hammer before attempting to crush it in a mortar and pestle. In some cases, it may be impossible to achieve complete disruption of the tissue. For example, due to its high content of connective tissue, breast tumor tissue is not amenable to either complete homogenization of fresh minced tissue or to thorough crushing of frozen tissue in liquid nitrogen. In such cases it may be advisable to go ahead with the isolation procedure after a reasonable effort at disruption, even if the tissue appears to still be mostly or partially intact.

See Ambion's Technical Bulletins #177, and #183 (available at www.ambion.com) for more information on tissue disruption.

Tissue or cells contain less RNA than expected

Expected yields of RNA vary widely between tissues. Researchers accustomed to working with tissues such as liver or kidney where RNA is plentiful may have unrealistically high expectations of RNA yields from tissues such as muscle, lung, or brain.

E. Contaminants in RNA; RNA Inhibits Enzymatic Reactions

DNA contamination

a. Precipitate the RNA with LiCl

LiCl precipitation (section III.D on page 15) will selectively precipitate RNAs longer than 200 nt, and will leave behind DNA, carbohydrates, and protein.

IMPORTANT

LiCl precipitation is not efficient when the RNA concentration is below 0.2 µg/µL.

b. Digest the prep with DNase I (RNase-free)

(See section VI.A on page 24). Ambion DNA-*free*™ DNase Treatment is perfect for this application, it includes guaranteed RNase-free DNase I and reaction buffer, as well as a unique DNase Inactivation Reagent. With DNA-*free*, DNase is inactivated quickly and easily without jeopardizing your RNA in a heat treatment, and without organic solvents and alcohol precipitation.

Other contaminants

Any of the following suggestions will further purify RNA after the RNAqueous procedure.

- Repeat RNAqueous purification (follow procedure for enzyme reactions: section *II.I. Enzyme Reactions: Lysate Preparation* on page 12).
- LiCl precipitate the RNA: LiCl precipitation (see section III.D on page 15) will selectively precipitate RNAs ≥200 nt, and will leave behind DNA, carbohydrates, and protein.

  IMPORTANT

  LiCl precipitation is not efficient when the RNA concentration is below 0.2 µg/µL.

- Digest with Proteinase K and SDS, and extract with phenol/chloroform (see sections VI.B on page 25, and VI.C on page 25).

VI. Optional RNA Clean-up Procedures

A. DNase I Treatment

Trace DNA contamination can be enzymatically removed using DNase I. Note that under optimal conditions, PCR can detect a single DNA molecule, so even DNase treatment cannot always guarantee removal of genomic DNA below the level detectable by PCR.

Ambion TURBO DNA-*free*™ Kit

The Ambion TURBO DNA-*free*™ Kit (P/N AM1907) includes Ambion TURBO DNase™ (patent pending), the first DNase I enzyme engineered for superior DNA digestion. TURBO DNA-*free* also simplifies the process of inactivating the DNase without the risk incurred by heating the RNA, or the inconvenience of extracting with phenol/chloroform. TURBO DNA-*free* is the method of choice for eliminating contaminating DNA prior to RT-PCR; to use TURBO DNA-*free*, follow the instructions provided with the product.

Using your own DNase treatment reagents

DNase digestion buffer

DNase treatment can be carried out in the buffer supplied or in a buffer recommended by the manufacturer of the enzyme; most restriction enzyme buffers can also be used. DNase I works well in a large range of salt and pH conditions. The enzyme requires magnesium (~5 mM) for optimal activity.

Amount of DNase

- To remove small amounts of DNA, DNase I should be used at approximately 10 Units/mL RNA.
- To treat severe DNA contamination, use 1 Unit DNase I per µg of contaminating DNA.

Incubation conditions

Incubate DNase digestions at 37°C for 30 min.

Inactivate DNase I by one of the following methods:

- Add EDTA to 5 mM, heat to 75°C for 10 min
- Add EDTA to 20 mM
- Extract with phenol/chloroform, and alcohol precipitate the RNA.

B. Proteinase K/SDS Treatment

Proteinase K is a nonspecific serine protease that is active in the presence of SDS or urea and over a wide range of pH/ salt concentrations and temperatures. Working concentrations of Proteinase K range from 50–250 µg/mL. Typical conditions for RNA-containing solutions are 0.1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.5% SDS, and 200 µg/mL Proteinase K incubated at 55°C for 30–60 min. The exact time of incubation and Proteinase K concentration depends on the amount of protein in the sample.

C. Phenol/Chloroform Extraction

Background

Phenol extraction is a method commonly used for deproteinization of nucleic acids. Most proteins are more soluble in phenol than in aqueous solutions. Conversely, nucleic acids are more soluble in aqueous solutions than in phenol. Centrifugation of the mixture will separate the phases; the lower phase is the organic phase and will contain the protein, usually as a white flocculent material at the interface. The upper aqueous phase will contain nucleic acids. Chloroform is mixed with phenol to enhance protein denaturation and phase separation. Chloroform in the phenol also improves its ability to remove lipids; isoamyl alcohol is added to prevent foaming.

Organic extraction protocol

The most rigorous way to perform a phenol/chloroform extraction is to first extract with buffer saturated phenol (Ambion P/N AM9710, AM9712), followed with a phenol:chloroform:isoamyl alcohol (Ambion P/N AM9720, AM9722) extraction, and finally, to extract the sample with chloroform:isoamyl alcohol. Instructions to prepare these reagents can be found in *Current Protocols in Molecular Biology* (Ausubel et al., eds.). Following is a detailed protocol that can be used for each of these organic extractions:

1. Adjust the aqueous volume of the sample to 100–200 µL with nuclease-free water or TE.
2. Add an equal volume of organic solvent solution, vortex for 2 min to mix thoroughly.
3. Spin at top speed in a room temperature microcentrifuge for 2 min.
4. Recover the aqueous phase by removing it to a new tube.

Precipitating the RNA

After the organic extractions are complete, the RNA can be precipitated by adding a concentrated ammonium acetate solution to a final concentration of 0.5M. Then add 2.5 to 3 volumes of 95–100% ethanol, mix thoroughly, and leave at –20°C for ≥15 min. Centrifuge at ≥10,000 x g for 15–20 min to recover the RNA.

VII. Appendix

A. Preparation of Phosphate Buffered Saline (PBS)

Concentration	Component	for 1 L
137 mM	NaCl	8 g
2.7 mM	KCl	0.2 g
10 mM	Na_2HPO_4	1.42 g
1.8 mM	KH_2PO4	0.25 g

Dissolve the components in about 800 ml dH_2O. Adjust the pH to 7.4 with HCl. Adjust the volume to one liter. Sterilize by autoclaving. Store at room temperature.

B. References

Ausubel FM, et al. (editors) *Current Protocols in Molecular Biology,* John Wiley and Sons.

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Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-Van Dillen PME, and Van Der Noordaa J (1990) Rapid and Simple Method for Purification of Nucleic Acids. *Journal of Clinical Microbiology.* **28.3**: 495–503

Cathala G, Savouret J, Mendez B, West BL, Karin M, Martial JA, and Baxter JD (1983). A Method for Isolation of Intact, Translationally Active Ribonucleic Acid. *DNA* **2**: 329–335.

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Huang Z, Fasco MJ, Kaminsky LS (1996) Optimization of DNase I Removal of Contaminating DNA from RNA for Use in Quantitative RNA-PCR. *BioTechniques* **20**:1012–1020.

Marko M, Chipperfield R, and Birnboim HC (1982). A Procedure for the Large Scale Isolation of Highly Purified Plasmid DNA Using Alkaline Extraction and Binding to Glass Powder. *Analytical Biochem.* **121**: 382–387.

Vogelstein B and Gillespie D (1979). Preparative and Analytical Purification of DNA from Agarose. *Proc. Natl. Acad. Sci. USA* 76: 615–619.

C. Safety Information

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT

For the MSDSs of chemicals not distributed by Applied Biosystems or Ambion, contact the chemical manufacturer.

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.)

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

D. Quality Control

Functional Testing

Mouse liver RNA is prepared according to the kit instructions. The RNA is checked for integrity and minimum yield requirements.

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.