USER GUIDE

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RNAqueous®-Midi Kit

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life technologies™

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RNAqueous®-Midi Kit

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Introduction

Product description

The RNAqueous®-Midi Kit employs a rapid, phenol-free, glass fiber filter-based procedure for RNA isolation. It is designed for use with midrange amounts of starting material, i.e 100–500 mg animal tissue samples or ~10^7–10^8 cultured eukaryotic cells.

Procedure overview

The RNAqueous-Midi Kit employs an RNA isolation method 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).

Most lysate, except that from glycogen-rich tissue, is first centrifuged to remove debris and particulate matter, and is then diluted with an ethanol solution. The prepared lysate is passed through a Syringe Filter Cartridge trapping the RNA on the filter. The Syringe Filter Cartridge is then washed 3 times to remove contaminants, and finally the RNA is eluted in a very low ionic strength solution.

The entire RNA purification procedure can be completed in about 10 minutes, once the clarified lysate has been prepared. The lysate preparation time varies from a minute or two, to as long as ~15 minutes depending on the sample type and the method used for disruption.

The recovered RNA is suitable for use directly in reverse transcription (RT) reactions, RT-PCR, Northern blot experiments, RNase protection assays, and most other common applications.

IMPORTANT! Very low molecular weight RNA is **not** recovered using the RNAqueous-Midi Kit, thus this procedure is not suitable for isolation of microRNAs, 5S ribosomal RNAs or tRNA.

Reagents provided with the kit and storage conditions

This kit provides reagents for 15 RNA isolations using 100–500 mg tissue sample or ~10^7–10^8 cultured eukaryotic cells per isolation.

Amount	Component	Storage
100 mL	Elution Solution	any temp§
30	Collection Tubes	room temp
15	Syringe Filter Units	room temp
36 mL	Water for 64% ethanol (Add 64 mL 100% ethanol before use)	4°C†
180 mL	Wash Solution #1‡	4°C††
252 mL	Wash Solution #2/3 Concentrate (Add 201.6 mL 100% ethanol before use)	4°C†
100 mL	Lysis/Binding Solution†	4°C
25 mL	Lithium Chloride Precipitation Solution	4°C
6 mL	Formaldehyde Load Dye‡	–20°C

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

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

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

†† 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

Required materials not provided with the kit

Reagents and supplies

- 100% ethanol: ACS reagent grade or equivalent
- Sterile, calibrated 3 mL disposable syringes with standard luer-lock fittings
- Calibrated 10 mL disposable syringes with standard luer-lock fittings (They do not need to be sterile.)
- 18 gauge 1.5 inch needles with standard luer-lock fitting
- 17 x 100 mm round bottom sterile disposable polypropylene tubes with loose-fitting dual position caps: for mixing lysates with ethanol and for heating Elution Solution in boiling water bath
- (optional) Plant RNA Isolation Aid (Cat. no. AM9690) is a high molecular weight polymer solution that can be used with the RNAqueous-Midi Kit to improve isolation of total RNA from plant tissues.

Tissue disruption equipment

- Liquid nitrogen, mortar and pestle, and dry ice: for grinding frozen tissue
- Electronic rotor-stator tissue homogenizers are recommended. Alternatively, manual homogenizers can be used; we recommend ground glass tissue grinders.

Other laboratory equipment

- Centrifuge, rotor, and tubes: for initial clarifying spin to remove debris from lysates (This equipment is needed for most solid tissue samples and in some cases for cultured cells.)
- Heat block or water bath set at 100°C.

Set-up and sample lysate preparation

Before using the kit for the first time

Prepare 64% Ethanol solution

Add 64 mL of 100% ethanol (ACS grade or equivalent) to the bottle labeled Water for 64% Ethanol, which contains 36 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 201.6 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.

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.

Amount of starting material

This procedure is designed for medium-scale RNA isolation from animal tissue or cells. The limiting factor in determining the maximum amount of starting material in this procedure is clogging of the RNAqueous-Midi Syringe Filter Cartridges. The following chart lists the amounts of different types of starting material recommended for RNA isolation with a single Syringe Filter Cartridge.

Material	Amount
Animal Tissue	• 100–500 mg for many tissues (Up to 1 g of some tissues can be processed. Sample size is typically limited by filter clogging rather than by exceeding its RNA binding capacity.) • ≤250 mg for glycogen-rich tissues such as liver
Mammalian Cells	10^7–10^8 cells, or 1–5 confluent 150 mm dishes

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 9). 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; the following table shows guidelines for 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

5. Thoroughly homogenize sample (100–500 mg) in 12–24 volumes of Lysis/Binding Solution

   Thoroughly homogenize 100–500 mg tissue samples in 12–24 volumes of Lysis/Binding Solution following the instructions in steps a.–c. on page 10 for samples stored in RNA*later*® Solution, or fresh or frozen samples.

   Determining the appropriate volume of Lysis Binding Solution

   - For most tissues, 12 volumes of Lysis/Binding Solution is sufficient. For example for 100 mg of tissue, use 1.2 mL of Lysis/Binding Solution, or for 500 mg of tissue, use 6 mL of Lysis/Binding Solution.
   - For glycogen-rich tissues such as liver from non-fasted animals, use a maximum of 250 mg tissue per filter, and homogenize in 24 volumes of Lysis/Binding Solution (i.e., 6 mL Lysis/Binding Solution for 250 mg tissue).

   a. Preparing samples stored in 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 b. (next). Extremely tough/fibrous tissues in RNA*later* Solution may need to be frozen and pulverized according to the instructions for frozen tissue (step c.) in order to achieve good cell disruption.

      If the samples were immersed in RNAlater 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.

b. Fresh tissue sample preparation (soft to medium consistency tissues)

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

ii. Aliquot 12–24 volumes or at least 200 µL of Lysis/Binding Solution into the homogenization vessel.

For example, if your sample weighs 500 mg, use 6–12 mL Lysis/Binding Solution (and 500 µL of Plant RNA Isolation Aid, if desired).

iii. Mince large samples (≥2 cm^2) rapidly in cold PBS, then remove the PBS before proceeding to the next step (see PBS recipe in Appendix A, "Preparation of Phosphate Buffered Saline (PBS)" on page 27).

iv. Drop samples into the Lysis/Binding Solution, and process to homogeneity. If available, use a motorized rotor-stator homogenizer.

c. 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.

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

ii. For each sample, place 12–24 volumes or at least 200 µL of Lysis/Binding Solution into 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.)

iii. 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 in a mortar and pestle.

iv. 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.

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

vi. 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.

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 2–6 mL Lysis/Binding Solution to 2 x 10^7 to 10^8 cells and lyse the cells

   a. Add Lysis/Binding Solution to cells following the guidelines in the table below 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.

Number of Cells	Volume Lysis/ Binding Solution
10^7	2 mL
2 x 10^7	4 mL
10^8	6 mL

   Frozen cells: Small frozen cell pellets can be lysed by adding Lysis/Binding Solution and using the pipet tip to disrupt the pellet while intermittently pipetting up and down to mix rapidly and thoroughly. Large cell pellets must be frozen in liquid nitrogen and crushed in a mortar and pestle as described for frozen tissue (step c. on page 10). If possible, it is better to lyse fresh cells as described above and freeze the lysate rather than freezing cells prior to lysis.

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

   c. It is a good idea to shear DNA in lysates by sonication or by passing the lysate through a 25 g syringe needle.

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

RNA isolation

Preparation of lysate for filter binding

1. Reduce the viscosity of the lysate if necessary

   The lysate should be somewhat viscous, but if it 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 an 18 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. If necessary, centrifuge the lysate at 4°C to remove debris

   IMPORTANT! Do not include this centrifugation when processing glycogen-rich tissue such as liver, because RNA (especially mRNA) could be pelleted with the cell debris.

   This step may not be necessary for samples smaller than ~100–200 mg, or for cells grown in tissue culture, if the lysate appears to be completely clear and free from particulate matter.

Centrifuge the lysate at 4°C, to remove particulate matter and cellular debris. The optimal conditions for the clarifying spin will vary depending on the type and amount of tissue used, and on how thoroughly it was disrupted/homogenized. Use the shortest time and lowest speed that effectively clarifies the lysate; centrifugation for ~10 min at 8000 x g is typically adequate for up to 500 mg of tissue. Optimal centrifugation conditions can vary from 3,000 x g for 5 min to 12,000 x g for 15 min.

3. Transfer the lysate to larger tube

   Transfer the lysate to a vessel that can hold over twice the volume of the lysate. When transferring the lysate, avoid contaminating it with pelleted material or with debris that may be floating on the surface. If necessary, remove floating debris with a cotton swab or pipet.

Filter binding, washing, and elution of RNA

1. Heat the Elution Solution to 100°C

   Preheat ~1.5–3 mL of Elution Solution per prep to 100°C in a disposable polypropylene tube with loose-fitting cap.

   IMPORTANT! Heat the Elution Solution in a tube with a loose-fitting cap to vent the contents during heating.

2. Add an equal volume of 64% Ethanol to the lysate

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

   IMPORTANT! If the preparation becomes highly viscous after addition of the ethanol due to high DNA content, shear the DNA by passing the preparation through an 18 gauge needle about 10 times or until the viscosity is significantly reduced.

3. Pass the mixture through a Syringe Filter Unit, then pass air through the filter 2–3 times

   a. Draw the lysate/ethanol mixture into a calibrated 10 mL syringe through an 18 gauge needle.

   b. Remove the needle and attach a Syringe Filter Unit to the syringe containing the sample. Slowly push the lysate/ethanol mixture through the filter unit into a waste receptacle.

      The mixture should drip out at a rate of ~3–5 drops per second.

      If the flow rate slows, continue filtering the solution at a slower rate. Do not use excessive force to pass the mixture through the filter.

      If the filter begins to clog, it is best to try to clear it before it becomes severely clogged by forcing air through the unit:

      IMPORTANT! To prevent damage to the filter, always remove the Syringe Filter Unit from the syringe before retracting the plunger.

Remove the Syringe Filter Unit from the syringe containing the lysate/ethanol mixture and use a clean 10 mL syringe to force air through the clogged Syringe Filter Unit several times, removing the filter from the syringe before retracting the plunger each time. Typically this process will expel white foam and unclog the filter.

This step can be repeated as necessary during filtration of the remaining lysate. See also section "Filter clogging" on page 18.

IMPORTANT! If the flow rate slows and then suddenly increases dramatically, the filter has probably torn. In this case, the yield of RNA will be extremely low.

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c. After all the lysate has been passed through the Syringe Filter Unit, force air through the filter ~2–3 times as described in the previous step. Usually some white foamy material will be expelled. Push air through the filter until no more foamy material is expelled.

4. Wash Syringe Filter Unit with an equal volume of Wash Solution #1

   a. Remove the Syringe Filter Unit from the syringe.

   b. Aspirate a volume of Wash Solution #1 equal to the volume of lysate/ethanol filtered in step 3. into the syringe. (Use the calibration marks on the syringe to measure the wash solution volume.)

   c. Reattach the filter unit to the syringe, and slowly pass the Wash Solution #1 through the filter into a waste receptacle.

      Note: The flow rate for may be slower than for the initial filtration of the lysate in step 3. If it is difficult to pass Wash Solution #1 through the filter, do not use excessive force; instead, force air through the filter to clear it, as described in step 3.b. (See also section "Troubleshooting" on page 18)

   d. Remove the filter and retract the plunger of the syringe. Reattach the filter and force air through it as in step 3.c.

5. Wash filter twice with a 70% volume of Wash Solution #2/3

   a. Remove the Syringe Filter Unit from the syringe.

   b. Aspirate a volume of Wash Solution #2/3 equal to 70% of the volume of lysate/ethanol filtered in step 3. into the syringe.

   c. Reattach the filter unit to the syringe, and pass the Wash Solution #2/3 through the filter into a waste receptacle.

      Wash Solution #2/3 should flow at a rate somewhat faster than the previous solutions (a slow stream rather than drop-wise).

   d. Repeat above steps to wash a second time with a 70% volume of Wash Solution #2/3.

6. Remove residual wash solution

   Remove the filter unit, retract the plunger of the syringe, reattach the filter, and vigorously force air through the filter to remove residual wash solution. Repeat until no additional droplets or fine spray is visible (~5–10 times). The outlet of the filter unit may then be blotted onto absorbent paper to remove residual fluid from the tip.

7. Elute RNA from the filter using 3 x 500 µL of 100°C Elution Solution
   a. Heat the Elution Solution to 100°C as described in step 1. on page 12, if you have not already done so.
   b. Attach the Syringe Filter Unit to a 3 mL syringe with the plunger removed.

      IMPORTANT! Do not allow the tip of the plunger to come into contact with any surface that has not been treated to remove RNases. A good place to rest the tip of the plunger is on the inverted cap of the Collection Tube.

   c. Add 500 µL of 100°C Elution Solution to the syringe. Insert the plunger and force the Elution Solution through the filter into a clean Collection Tube.

      Note: The volume of Elution Solution used is not critical. Less solution can be used to recover the RNA in a more concentrated solution. It is important, however, to elute the RNA using three aliquots of Elution Solution for maximum recovery.

   d. Remove the Syringe Filter Unit, remove the plunger, and reattach the filter unit. Repeat the elution two more times, forcing the 500 µL aliquots of hot Elution Solution into the same Collection Tube.
   e. After the third aliquot, force air through the filter and recover the last traces of eluate. Cap the tube and mix the eluted RNA briefly.
   f. If desired, the elution step may be repeated. The amount of RNA recovered in a second set of elutions depends on the type and amount of tissue or cells used. RNA bound to the washed filter will be stable for the time needed to assess the yield of RNA in the first and/or second elution by gel electrophoresis or spectrophotometer readings. If an appreciable amount of RNA is recovered in the second elution, a third elution may be beneficial.

(Optional) Precipitate with LiCl

Lithium chloride precipitation removes carbohydrates and gross DNA contamination.

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

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.
7. Resuspend the pellet (which may be invisible) in the desired amount of Elution Solution. Solubilization of the pellet is facilitated by heating and vortexing.

Assessing RNA yield and quality

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 (Cat. no. 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.

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

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

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

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

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

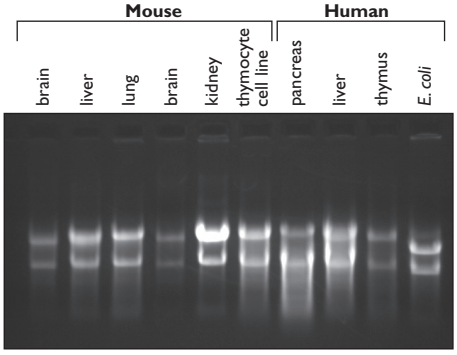
Load the gel and electrophorese at 5–6 V/cm until the bromophenol blue (the faster-migrating dye) has migrated at least 2–3 cm into the gel, or as far as 2/3 the length of the gel.

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 1 shows a typical denaturing agarose gel containing RNA isolated with the RNAqueous-Midi 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 1 RNA Isolated Using the RNAqueous®-Midi Kit. RNA was analyzed as described above on a 1% agarose/formaldehyde gel. The amount of each preparation analyzed ranged from 5 µL to 15 µL; the total volume of the preparations was 1.5 mL or 3 mL. Since different amounts of tissue were used, no direct comparisons can be made about the relative yields of RNA from the different preparations.



Troubleshooting

Filter clogging

Tissue lysis/disruption is incomplete

1. Process tissue to as small a fragment size as possible prior to addition to the Lysis/Binding Solution.

   Grind smaller snap-frozen tissue chunks under liquid nitrogen using a mortar and pestle.

   Fold large pieces of frozen tissue in a heavy-duty foil pouch and crush with several vigorous blows from a hammer (it's a good idea to put the foil-wrapped sample on the floor instead of the lab bench before hammering it, and to further cushion it with several layers of newspaper).

   Fresh tissue should be cut into small pieces with a scalpel.

   Note: The Lysis/Binding Solution effectively inactivates cellular RNases even at room temperature, thus it is generally not necessary to keep the sample on ice during the homogenization step. However, it is essential that frozen tissue remain completely frozen prior to adding it to the Lysis/Binding Solution.

2. Homogenize tissue completely in Lysis/Binding Solution

   Electronic rotor-stator homogenizers are recommended.

   Maximize disruption efficiency by using a narrow, deep vessel to hold the preparation.

   Visually inspect the lysate to Monitor the extent to which the tissue disruption is complete by carefully inspecting the lysate; visible particles are indicative of suboptimal homogenization.

Lysate contains debris

- Optimize the centrifugation to clarify the lysate (step 2. on page 11).
  - The time and centrifugal force needed to remove cell debris depends on the type and amount of tissue being processed. For some tissues (e.g., brain), especially small samples (100–200 mg), it is not always necessary to centrifuge the lysate. On the contrary, lysates from kidney and pancreas will almost always require clarification.

    IMPORTANT! This centrifugation step must be done prior to mixing the preparation with 64% ethanol. Once mixed with ethanol, centrifugation will precipitate the RNA.

  - The optimum centrifugation conditions can be determined empirically by centrifuging the lysate for a given time and speed, then removing a small aliquot (~1 mL) and spinning it for an additional ~5 min at maximum speed in a microcentrifuge. If additional material pellets or floats out of the small aliquot of clarified lysate, the remaining preparation should be centrifuged again at a higher centrifugal force.
  - Avoid centrifuging the lysate for a longer time and/or at higher speed than needed to remove debris, because this may decrease RNA yield.
  - Take care that material floating on the surface of the lysate does not stick to the pipet when the lysate is transferred to the fresh tube, so that it does not contaminate the clarified preparation.

- The greater the amount of tissue processed per preparation, the more stringent the requirements for the clarifying spin. In other words, a 5 min spin at 5,000 rpm may be sufficient for filtering 2.4 mL of lysate (made from 200 mg of tissue), but a longer, harder spin may be needed to prevent filter clogging when processing 12 mL of lysate (made from 1 g of the same tissue).
- Once optimal conditions have been established for a given tissue, record them for future reference. However, different batches of the same tissue or even different aliquots of the same batch of lysate may require different conditions for the clarifying spin. This variation may be due to differences in the extent to which debris has sedimented in the lysate (i.e., the lysate at the bottom of the tube may contain more debris than the lysate in the upper part of the tube).

Lysate is too viscous

- Shear the DNA by forcing the lysate through an 18 gauge needle.

  If the lysate is extremely viscous, or has blobs of viscous, gelatinous material, it probably has a high DNA concentration. This is more likely with lysates made from cells grown in culture than from solid tissue.

  Reduce lysate viscosity by vigorously and repeatedly aspirating the preparation up into a syringe and forcing it out through an 18 gauge needle in order to shear the DNA into smaller fragments.

  Sometimes the lysate does not become noticeably viscous until it is mixed with ethanol. The shearing step can be done either before or after the lysate is mixed with ethanol, but it is more convenient to do it before, when the volume of the preparation is smaller.

Recovering from a clogged filter

1. Try to clear the filter so that the remaining lysate can be passed through it.

   Attach the clogged filter to a second 10 mL syringe with the plunger retracted, and vigorously pump air through it several times. This is most effective if done before the filter is severely clogged, i.e., when the flow rate has slowed but not stopped.

   IMPORTANT! Be sure to remove the filter before retracting the syringe each time air is forced through, to prevent the filter from tearing.

   This step can be repeated as necessary during filtration of the remaining lysate.

   Filters that clog during filtration of the Wash Solution #1 can also be cleared by pumping air through them.

2. Use a second filter for the remaining lysate.

3. Discard the remaining unfiltered lysate and try to recover the RNA from the clogged filter.

   IMPORTANT! If the flow rate slows and then suddenly increases dramatically, it indicates that the filter has torn. In this case, the RNA yield will be extremely low.

   Continue the procedure as usual. This is usually successful as long as the filter has not been seriously clogged or torn.

To prevent serious clogging or tearing, do not attempt to force the remaining lysate through the filter once it is apparent that the rate of filtration has slowed significantly.

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.

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-Midi 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.

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.

Low RNA yield

Poor tissue disruption

See Section "Filter clogging" above for tissue disruption suggestions.

Overcentrifugation of lysate in the clarification step

To minimize RNA loss in the clarification spin, use the minimal time and centrifugal force required to remove cellular debris. See Section "Lysate contains debris" on page 18 for instructions on optimizing this step of the procedure.

Poor elution of RNA from glass fiber filter

Keep the Elution Solution as hot as possible.

Use Elution Solution soon after it is removed from the hot plate or heat block.

To slow the cooling process, keep the tube of Elution Solution in the beaker of hot water used as water bath while performing the elution.

Elute RNA with several smaller-volume elutions

Pass several smaller aliquots of hot Elution Solution sequentially through the filter, rather than a single larger aliquot (i.e. elute in 3 x 0.5 mL of solution per tube, rather than 1 x 1.5 mL).

Rarely, more RNA may be recovered in the second elution than in the first; in this case, a third elution should be performed to maximize recovery of RNA.

Normal variation in RNA content of different tissues/cells

Expected yields of RNA vary widely between tissues. For example, liver typically yields ~3–5 fold more RNA per unit mass than brain. Often a lower than expected yield of RNA will still be sufficient for the intended application. If necessary, the RNA can be concentrated by ethanol precipitation.

Supplemental Information

Optional RNA clean-up procedures

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 (Cat. no. AM1907) includes Ambion® TURBO DNase™, 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. TTURBO 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.

For reasons that are not clear, RNA samples treated with DNase sometimes fail to leave the well when they are assessed on formaldehyde gels. In our experience, adding EDTA to a concentration equal to two-fold the Mg++ concentration of the DNase digestion buffer allows the RNA to migrate normally in agarose gels.

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.

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 (Cat. nos. AM9710, AM9712), followed with a phenol:chloroform:isoamyl alcohol (Cat. nos. 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.

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	Na2HPO4	1.42 g
1.8 mM	KH2PO4	0.25 g

Dissolve the components in about 800 mL dH2O. Adjust the pH to 7.4 with HCl. Adjust the volume to one liter. Sterilize by autoclaving. Store at room temperature.

Related products available from Life Technologies

RNA*later*® Solution Cat. nos. AM7020, AM7021	RNA*later*® Tissue Collection: RNA Stabilization Solution is an aqueous sample collection solution that stabilizes and protects cellular RNA in intact, unfrozen tissue and cell samples. RNA*later* Solution eliminates the need to immediately process samples or to freeze samples in liquid nitrogen. Samples can be submerged in RNA*later*® Solution 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 Cat. nos. AM7030, AM7031	RNA*later*®-ICE Frozen Tissue Transition Solution is designed to make it easier to process frozen tissue samples for RNA isolation. Simply drop frozen tissues into RNA*later*®-ICE Solution and store overnight at –20°C. Once tissues are treated they can be easily processed using standard RNA isolation procedures.
RNase*Zap*® Solution Cat. nos. AM9780, AM9782, AM9784	RNase*Zap*® RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*® Solution.
RNase-free Tubes & Tips	Ambion® RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free.
TURBO DNA-*free*™ Kit Cat. no. AM1907	The TURBO DNA-*free*™ Kit employs TURBO™ DNase, 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.
Millennium™ Markers and BrightStar® Biotinylated Millennium™ Markers Cat. nos. 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.
Electrophoresis Reagents	Life Technologies offers gel loading solutions, agaroses, acrylamide solutions, powdered gel buffer mixes, nuclease-free water, and RNA and DNA molecular weight markers for electrophoresis..

Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:
Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.

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

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials.

Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/ 29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/ handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/ csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

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Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/sds**

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

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