RNAqueous®-4PCR Kit

Kit for Isolation of DNA-free RNA

Part Number AM1914

RNAqueous®-4PCR Kit

(Part Number AM1914)

Protocol

P/N 1914M Revision D **Revision Date: September 6, 2010**

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

Literature Citation: When describing a procedure for publication using this product, please refer to it as the RNAqueous®-4PCR Kit.

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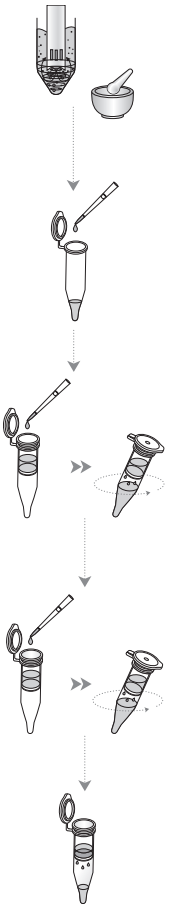
I. Introduction

A. Procedure Overview

The Ambion® RNAqueous®-4PCR Kit is for isolating total RNA from small tissue samples (0.5–75 mg) or cells (from ~100 cells to ~1 x 10^7 cells). The method used is based on disruption of tissue or cells in a solution containing guanidinium thiocyanate, a strong chaotropic denaturant which lyses cell membranes and rapidly inactivates cellular ribonucleases (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987). One option for tissue disruption is to use the disposable pestles included in the kit. Cultured cells can typically be disrupted by simply vortexing the cell pellet in the guanidinium lysis solution. The sample lysate is then mixed with an ethanol solution, and applied to a silica-based filter which selectively and quantitatively binds mRNA and the larger ribosomal RNAs; very small RNAs such as tRNA and 5S ribosomal RNA are not quantitatively bound. The filter is then washed to remove residual DNA, protein, and other contaminants, and the RNA is eluted in nuclease-free water containing a trace amount of EDTA to chelate heavy metals. The silica filter is housed in a small cartridge which fits into the RNase-free microfuge tubes supplied with the kit. The sample lysate, wash solutions, and elution solution are moved through the filter by centrifugation or vacuum pressure. The entire RNA isolation procedure takes about 10 min. After elution from the filter the RNA may be treated with the ultra-pure DNase 1 provided with the kit to remove trace amounts of DNA. Finally, the DNase and divalent cations are removed by a reagent also provided with the kit. DNase treatment and inactivation takes about 30 min. The RNA recovered can be used for most common applications, including cDNA synthesis, RT-PCR, in vitro translation, and blot hybridization. Yields of RNA will vary according to the type and amount of sample, but will typically fall in the range of 1–10 µg per mg tissue, or ~1 µg per 1 x 10^5 cells in culture.

Figure 1. RNAqueous®-4PCR Kit Procedure



1. Start with 0.5–75 mg tissue or 10^2–10^7 cells

2. Disrupt samples in Lysis/Binding Solution

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

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

5. Wash with 700 µL Wash Solution #1

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

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

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

B. Materials Provided with the Kit and Storage Conditions

Reagents for 30 RNA isolations from ≤75 mg samples.

The minimum storage requirements for the kit components are shown below. For convenience, the components marked for room temperature (RT) storage can be stored at 4°C if desired. Storage at –20°C should be in a non frost-free freezer.

Amount	Component	Storage
600 µL	10X DNase 1 Buffer 100mM Tris/25mM $MgCl_2$ /1mM $CaCl_2$	–20°C
45 µL	DNase 1 (RNase-free) 2 units/µL	–20°C
1 mL	Formaldehyde Load Dye*	–20°C
100 µL	Linear Acrylamide, (5 mg/mL)	–20°C
50 µL	Positive Control Primers (mixture of 5 µM forward and reverse)	–20°C
1 mL	5M Ammonium Acetate	–20°C
35 mL	Water for 64% Ethanol (must be diluted with 22.4 mL ethanol)	4°C
600 µL	DNase Inactivation Reagent	4°C
30 mL	Lysis/Binding Solution†	4°C
25 mL	Wash Solution #1‡	4°C‡
35 mL	Wash Solution 2/3 Concentrate (must be diluted with 28 mL ethanol)	4°C‡
3 each	Pestles for tissue disruption	room temp
30 each	Filter Cartridges	room temp
60 each	Collection Tubes	room temp
25 mL	Elution Solution (0.1 mM EDTA)	any temp**

* This reagent contains formamide and formaldehyde; both are potentially hazardous substances, and formaldehyde is a known carcinogen. Handle cautiously.

† These reagents contain guanidinium thiocyanate; this is a potentially hazardous substance that 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.

C. Required Materials Not Provided with the Kit

Reagents

100% ethanol, ACS grade

Phosphate buffered saline (PBS)

Equipment and supplies

Heat block

Microcentrifuge

1.5 mL RNase-free microfuge tubes, pipettors and tips

D. Related Products Available from Applied Biosystems

High Capacity cDNA Reverse Transcription Kit P/N 4322171, 4368813, 4374967, 4368814, 4374966	The Applied Biosystems High Capacity cDNA Reverse Transcription 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.
RNA*later*® Solution P/N 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.
RNase*Zap*® Solution P/N 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 see our web or print catalog	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/prod/tubes) for specific information.
PBS 10X, pH 7.4 P/N AM9624, AM9625	(Phosphate Buffered Saline) This high quality prepared solution is nuclease-free, and is rigorously tested for lot to lot consistency.
RETROscript® Kit P/N AM1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™, this kit provides reagents, controls and protocols for reverse transcription and PCR. Both oligo(dT) and random primers for cDNA priming are included, as is RNase inhibitor.
*SuperTaq™ P/N AM2050, AM2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
†SuperTaq™ Plus P/N AM2054, (50U) P/N AM2056, (250U)	Extended Range Thermostable DNA Polymerase. Super Taq Plus has a proof reading activity, and produces significantly higher yields of PCR products than ordinary Taq polymerase (includes 10X buffers and dNTPs)
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.

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II. RNAqueous-4PCR Kit Procedure

A. Before Using the Kit for the First Time

Prepare 64% Ethanol solution

Add 22.4 mL of 100% ethanol (ACS grade or equivalent) to the bottle labeled Water for 64% Ethanol, which contains 12.6 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 28 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. 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.

D. RNA Isolation Procedure

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. Start with 0.5–75 mg tissue or 10^2–10^7 cells

a. Mammalian cells

Ideally cells in culture should be processed fresh (i.e. not frozen). If you need to store cells before RNA isolation, Ambion RNA*later*® Solution can be used for this purpose. If you want to isolate RNA from frozen cell pellets, they should be ground to a powder in liquid nitrogen before adding the Lysis/Binding Solution. Instructions for handling fresh cell culture samples follow.

i. Cells grown in suspension
Pellet the cells at low speed, and discard the culture medium.

ii. Adherent cells
Aspirate and discard the culture medium from the culture vessel.

b. Tissue samples

See Figure 2 for estimation of mass values for small pieces of tissue. The recommended procedure for storage of tissue prior to RNA isolation is to put dissected tissue directly into 5 volumes of RNA*later* tissue collection/RNA stabilization solution (Ambion P/N AM7020). Store the tissue, immersed in RNA*later*, at 4°C for short-term storage (up to 1 week), or at –20°C for long-term storage.

Alternatively, fresh tissue can used provided it is processed immediately, or tissue can be snap-frozen in liquid nitrogen.

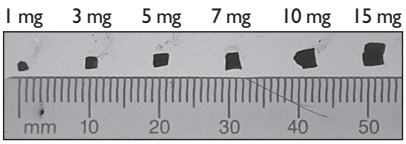

Figure 2. Estimating Mass of Small Tissue Samples

Pieces of mouse liver tissue weighing from 1–15 mg, were placed on a 1 mm scale ruler, and photographed. All pieces are approximately 1–2 mm thick. Most other soft tissues of are of similar density.

2. Disrupt samples in Lysis/Binding Solution

a. Mammalian cells

Add 100–500 µL of Lysis/Binding Solution and vortex vigorously. The exact volume of Lysis/Binding Solution is not critical, but in general, low-end volumes are used for fewer cells and high-end volumes are used for more cells. For example 100 µL of Lysis/Binding Solution is appropriate for 100–1,000 cells but, 500 µL should be used for 1 x 10^7 cells.

Vortex vigorously to lyse cell pellets. Continue vortexing until the lysate is homogenous.

Adherent cells should be lysed by vigorous pipetting and/or by using a scraper to mix the cells with the Lysis/Binding Solution. Transfer the lysate to a tube, and vortex well to completely homogenize the sample.

If the sample size is close to the 10^7 cell maximum, it may be a good idea to clarify the lysate by centrifugation for 2–3 min at top speed in a microcentrifuge. This can help avoid clogging of the filter in subsequent steps.

b. Solid tissue

Blot the tissue on a piece of absorbent paper to remove excess moisture (the paper need not be RNase-free). Measure or estimate the weight of the tissue. (see Figure 2)

Add 10–12 volumes per tissue mass of Lysis/Binding Solution and disrupt thoroughly. The exact volume of Lysis/Binding Solution used is not critical but 100 µL is the minimum volume recommended for ease of handling. The ratio of Lysis/Binding Solution:tissue mass may be adjusted to give optimal results for different tissues.

Thoroughly disrupt the tissue in Lysis/Binding Solution.

i. Tissue pieces that weigh less than ~25 mg

These may be disrupted in a 1.5 mL microfuge tube using one of the small plastic pestles provided in the kit. (Note, three pestles are provided. They may be washed and re-used for multiple preps.)

When using the pestles provided, the tissue may be disrupted using less Lysis/Binding Solution for optimal contact of the pestle and tissue. Add the remaining Lysis/Binding Solution as soon as the tissue is homogenized to minimize the time that the tissue is exposed to the reduced volume of Lysis/Binding Solution. This may not be practical for tissues that are very high in nuclease, such as pancreas.

ii. For larger amounts of tissue
Disruption can be carried out in small conical ground-glass homogenizers available from most large scientific supply catalogs. Other options for disruption are ceramic mortar and pestles and motorized rotor-stator homogenizers.

If the sample size is close to the 75 mg maximum, it may be a good idea to clarify the lysate by centrifugation for 2–3 min at top speed in a microcentrifuge. This can help avoid clogging of the filter in subsequent steps.

iii. Frozen tissue
Frozen tissue should generally be ground to a powder with liquid nitrogen before mixing with the Lysis/Binding Solution. Very small pieces of frozen tissue (<25 mg) may not require grinding in liquid nitrogen.

3. 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.

4. 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 5. 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.

5. 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.

6. 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.

7. 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).

8. 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.


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.

E. (optional) DNase 1 Treatment and DNase Inactivation

No RNA isolation procedure can guarantee the complete removal of trace amounts of DNA below the limit of detection by RT-PCR. DNase 1 treatment is recommended for RNA that will be used for RT-PCR because it effectively removes trace DNA contamination from RNA. It is especially important that no DNA is present in RT-PCRs using primers that do not flank introns, or for genes that have processed pseudogenes, because the RT-PCR products from RNA and contaminating DNA cannot be distinguished by size in these cases.

The DNase I must be removed from RNA that will be subjected to RT-PCR because it could degrade DNA made in the PCR. The DNase Inactivation Reagent also removes divalent cations introduced by the DNase 1 Buffer. This is important because divalent cations can degrade RNA at temperatures typically used for RNA denaturation prior to reverse transcription.

1. Add 0.1 volume of 10X DNase 1 Buffer and 1 µL of DNase 1

Mix gently.
If the RNA was eluted in >100 µL Elution Solution, use 1 µL DNase I per 100 µL RNA (in Elution Solution). If the RNA was eluted into ≤100 µL Elution Solution, use 1 µL DNase I per sample.

2. Incubate 15–30 min at 37°C

The RNA should now be free from contaminating DNA. The next few steps remove the DNase from the prep.

3. Add 0.1 volume DNase Inactivation Reagent

The DNase Inactivation Reagent is supplied as a slurry and must be dispersed by vortexing or pipetting prior to use.

The DNase Inactivation Reagent may become difficult to pipet after being used multiple times, due to depletion of fluid from the interstitial spaces. If this is the case, add a volume of nuclease-free water equal to about one-tenth the volume of the remaining Inactivation Reagent, and re-vortex to make a pipettable slurry.

4. Mix gently, incubate 2 min at RT

Flick the tube gently to disperse the DNase Inactivation Reagent in the reaction. Flick the tube once more during the 2 min incubation to re-disperse the reagent.

5. Centrifuge the tube at 10,000 x g for ~1 min to pellet the DNase Inactivation Reagent

In general it is not necessary to remove the RNA solution from the pelleted DNase Inactivation Reagent for short term storage, but avoid transferring any of the DNase Inactivation Reagent into subsequent reactions. For long term storage of RNA, we recommend removing the RNA to a new tube.


NOTE

If the DNase Inactivation Reagent is left in the tube, leave it undisturbed when removing aliquots of RNA. If necessary, centrifuge briefly to make a tighter pellet. Although we have not seen any inhibition of RT-PCR in the presence of deliberately carried-over DNase Inactivation Reagent, we recommend avoiding introduction of the DNase Inactivation Reagent into PCR.

F. (optional) Concentrate the RNA by Precipitation

The purpose of this precipitation is to concentrate the RNA, so that a larger mass amount of RNA can be added to subsequent reverse transcription reactions.

1. Add 0.1 volume 5 M Ammonium Acetate and 0.01–0.02 volumes Linear Acrylamide

Vortex briefly to mix. Linear Acrylamide is used to increase the recovery of small amounts of nucleic acids in alcohol precipitation. It can be omitted if the sample size was close to the maximum, i.e. > 50 mg tissue, or $>10^5$ cells.

2. Add 2–2.5 volumes 100% ethanol, mix well, place at ≤20°C for ≥25 min

This step may be prolonged indefinitely for user convenience.

3. Centrifuge at top speed 15 min

This will pellet the RNA. The centrifugation should be at RCF ≥10,000 x g. Remove the supernatent by gentle aspiration.

Re-spin the tube for several seconds and remove the residual fluid using a small-bore pipet.

4. Resuspend the RNA in ~15–20 µL Elution Solution

The amount of Elution Solution used is not critical, it can be increased or decreased to suit the subsequent experiment. The RNA can alternatively be resuspended in the solvent of your choice.

To aid in solubilization of the pellet, incubate the preparation at 65°C for ~5 min with intermittent gentle vortexing.

III. Assessing Yield and Quality of RNA

A. Quantitation and Assessment of RNA Purity by UV Absorbance

The concentration and purity of an RNA solution can be determined by measuring its absorbance at 260 and 280 nm 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.

Concentration

An A_{260} of 1 is equivalent to 40 µg RNA/mL. Find the concentration in µg/mL by multiplying 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.

Purity

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

B. RNA Electrophoresis on Denaturing Agarose

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.

b. 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.

Table 1. 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.)

The 28S and 18S ribosomal RNA bands should be fairly sharp, intense bands with mobilities of about 5 kb and 2 kb, respectively. 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 larger ribosomal RNA band. Degradation of the RNA will be reflected by smearing of ribosomal RNA bands. The figure below shows a typical formaldehyde agarose gel containing total RNA from several mouse tissues, isolated using the RNAqueous-4PCR kit.

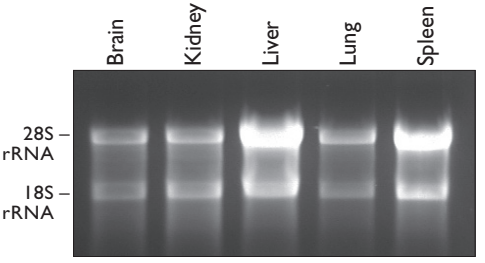

Figure 3. Mouse RNA isolated using RNAqueous-4PCR

Total RNA was isolated from 3 mg samples of the indicated mouse tissues. RNA was eluted in 50 µL, and 10 µL was run on a denaturing agarose gel in the presence of ethidium bromide.

IV. Troubleshooting

A. Using the Positive Control Primers

1. Positive Control Primers

Positive Control Primers for PCR (a mixture containing 5 µM each of forward and reverse primers) are included so that RNA obtained using the RNAqueous®-4PCR kit can be assessed in RT-PCR.

The Positive Control Primers amplify a ***361 bp***, highly conserved region of a constitutively expressed "housekeeping" gene, rig/S15, which encodes a small ribosomal subunit protein (Inoue et al. 1987; Kitagawa et al. 1991). The primers correspond to the human S15 sequence, and they should work well with human, mouse, hamster, and rat mRNA, and probably with many other vertebrate S15 sequences as well. Because it is a constitutively expressed message, the S15 RNA will be present in RNA isolated from any tissue.

Positive Control Primer sequences: PCR product size = 361 bp	
forward primer	5'-TTCCGCAAGTTCACCTACC
reverse primer	5'-CGGGCCGGCCATGCTTTACG

2. Uses of the Positive Control Primers

Check your RNA

The Positive Control Primers can be used to verify that an RNA preparation can support RT-PCR (if the RNA contains an S15 mRNA with the Positive Control Primer binding sites).

Check for DNA contamination in RNA

The Positive Control Primers span two introns; the forward primer binds in the second exon, and the reverse primer binds in the fourth exon. Despite this, they amplify a product of the same size from mouse and human genomic DNA and from cDNA. This is presumably due to the presence of processed pseudogenes in these genomes. This makes the Positive Control Primers useful for detecting DNA contamination in RNA preps used for RT-PCR. A 2–5 µL aliquot of mouse or human RNA can be added directly to a PCR (without prior reverse transcription); if a product is amplified with the Positive Control Primers, then genomic DNA is probably present in the RNA.

3. Positive control RT-PCR protocol

a. Reverse transcription

Use either oligo(dT) or random decamers as first-strand primers, with ~30 to 50% (usually 5–10 µL) of the RNAqueous-4PCR RNA preparation as template, in a 20 µL reaction. Use standard buffers, nucleotide concentrations, and enzymes for reverse transcription. Heat denature the RNA before the reverse transcription. A typical reverse transcription protocol is provided below.

i. Mix the following in an RNase-free microfuge tube:

Amount	Component
~5–10 µL	total RNA
2 µL	Oligo(dT) or Random Decamers*
to 12 µL	dH_2O, nuclease-free

* This volume is for a 50 µM stock of primer; use the amount of primer that will give a final concentration of 5 µM (final reaction volume is 20 µL)

ii. Mix, spin briefly, heat 3 min at 70–85°C.

iii. Remove tube(s) to ice; spin briefly, replace on ice.

iv. Add the remaining RT components:

Amount	Component
2 µL	10X RT Buffer
4 µL	dNTP mix (2.5 mM each)
1 µL	RNase Inhibitor
1 µL	Reverse Transcriptase (100–200 U)
to 20 µL	Nuclease-free Water

v. Mix gently, spin briefly. Incubate at 42–44°C for one hour.

vi. Incubate at 92°C for 10 min to inactivate the Reverse Transcriptase.

b. Amplification

Use ~5 µL of the reverse transcription reaction and 2.5 µL of the Positive Control Primers in a 50 µL PCR, according to standard protocols. Be sure to include a no-RT control that contains an equivalent mass amount of RNA that was not reverse transcribed. Use the thermocycle profile shown below:

Heat to 95°C: 1 min

Cycle 30–35X:
94°C - 30 sec, 55°C - 30 sec, 72°C - 30 sec

Final Extension: 72°C: 5 min

The expected product is 361 bp.


NOTE

This thermocycle profile works well using thin-walled 0.5 mL tubes, with a Perkin-Elmer Model 9600 Thermal Cycler. The profile may need to be adjusted to give optimal results with other instruments and/or reaction vessels. In particular, thermal cyclers with very rapid ramp times may require slightly longer cycle times, and machines with no ramp time (such as Stratagene's RoboCycler®) may require 1 min at each temperature in the PCR cycle.

c. Visualization of the results
Run ~10 µL of the PCRs on a 2–2.5% native agarose gel in the presence of ethidium bromide.

4. Expected result

The positive control RT-PCR should yield a single ***361 bp*** product that is easily visible.

B. Filter Clogging

Difficulty moving the lysate or wash solutions through the filter is usually seen when processing relatively large amounts of solid tissue, and is more pronounced with certain types of tissue (e.g. liver, kidney).

1. Homogenize samples more thoroughly

Filter clogging can often be reduced by more thoroughly homogenizing the sample, for example by using a motorized rotor-stator homogenizer instead of a manual tissue grinder, and/or by processing the tissue for a longer time.

2. Reduce sample viscosity

It may help to reduce the viscosity of the lysate by sonication or by passing it through a syringe needle to shear DNA.

3. Do a clarifying spin

If filter clogging is still a problem after trying the previous suggestions, use a clarifying spin at step II.D.2 on page 7 prior to the addition of 64% Ethanol, to remove debris and insoluble material. Spin the lysate at 10,000 x g for 1 min, and remove the clear supernatent to a fresh tube before adding the 64% Ethanol. It is important to do this clarifying spin before adding the ethanol to avoid loss of RNA at this step. Avoid clarifying the lysate at too high a centrifugal force or for too long a time, as this may also lead to loss of RNA.

C. Contamination of the Recovered RNA with DNA

This problem is usually observed as an amplified product in the minus-reverse transcriptase control in RT-PCR experiments. It can also be observed as the presence of high-molecular weight ethidium-staining material visible on an agarose gel, especially if relatively large amounts of tissue were used. The problem is more pronounced with certain types of tissue, for example, kidney and spleen.

DNA contamination can be eliminated by doing the DNase treatment. If DNA contamination is severe (i.e. visible on an agarose gel), it may be necessary to increase the amount of DNase 1 to 2–3 µL in step II.E.1 on page 10, and/or to prolong the DNase incubation step to 45 min to 1 hour. Also, be sure to use the DNase 1 Buffer included in the kit in the DNase 1 reaction for best results.

D. RT-PCR Yields Little or No Product

Nested PCR

Low yields of RT-PCR products can often be improved by doing a second PCR, using primers that are "nested" within the first-round product. If it is not convenient to use nested primers which are completely internal to the first-round primer binding sites, they can be "partially nested", i.e. their sequence(s) may partially overlap that of the first-round primers, but the 3' ends of the nested primers should extend past the 3' ends of the original primers. Even when the yield of the first-round amplification product is too low to be detected by ethidium staining, it may often be used successfully as a template in the nested reaction to generate a product which is visible by ethidium staining.

For the nested PCR, use an aliquot of the first-round product (~1–10 µL) in a 50 µL PCR, using the nested primers. The thermocycle profile may need to be re-optimized for the nested primers, for example the annealing temperature may need to be adjusted if the G/C content of the nested primers is significantly different from the first-round primers. Besides improving the yield of product, an additional benefit of nested reactions is that the specificity of the amplified product may be increased, since any unwanted products amplified in the first round will not generally contain the primer binding sites for the nested primers.

Redesign PCR primers

Yields of RT-PCR products may also be improved in some cases by re-designing the primers used for the PCR step. The use of computer software programs for efficient primer design is highly recommended.

Change the primer used for the reverse transcription

Another suggestion for improving yields is to use random oligonucleotide first-strand primers (for example, random decamers or random hexamers), or to use oligo dT as first strand primers, rather than using the reverse PCR primer, to prime cDNA synthesis. The Positive Control Primers provided with the kit are very efficient and may be used to assess the ability of most RNA preparations to serve as template in RT-PCR (see section IV.A on page 15).

V. Appendix

A. References

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Shiga K, Yamamoto H, and Okamoto H (1990). Isolation and characterization of the human homologue of *rig* and its pseudogenes: The functional gene has features characteristic of housekeeping genes. *Proc. Natl. Acad. Sci. USA* **87**:3594–3598.

B. Quality Control

Functional testing

Mouse kidney RNA is prepared according to the kit instructions, including the DNase treatment and inactivation. The RNA is checked for integrity and minimal yield requirements. RT-PCR is performed using the Positive Control Primers, and products are analyzed for size.

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.

C. Safety Information

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 goggles, 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.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to new customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining the MSDS

To obtain Material Safety Data Sheets (MSDSs) for any chemical product supplied by Applied Biosystems or Ambion:

- At **www.appliedbiosystems.com**, select **Support**, then **MSDS**. Search by chemical name, product name, product part number, or MSDS part number. Right-click to print or download the MSDS of interest.
- At **www.ambion.com**, go to the web catalog page for the product of interest. Click **MSDS**, then right-click to print or download.
- E-mail (MSDS_Inquiry_CCRM@appliedbiosystems.com) or telephone (650-554-2756; USA) your request, specifying the catalog or part number(s) and the name of the product(s). We will e-mail the associated MSDSs unless you request fax or postal delivery. Requests for postal delivery require 1–2 weeks for processing.

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