RNAqueous®-96 Kit

(Part Number AM1920)

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I. Introduction

A. Overview of the Procedure

The RNAqueous®-96 Kit is designed for rapid high-throughput purification of total RNA from cells or tissue. The method is based on disrupting samples in a lysis solution containing guanidinium thiocyanate and other ingredients for rapid solubilization of cellular membranes and simultaneous inactivation of cellular nucleases (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987). After lysis, the sample is mixed with an ethanol solution, and is passed over a silica filter contained in the wells of a 96-well plate. The RNA in the sample binds to the silica filter while other cellular components pass through. The RNA may be treated with DNase while bound to the filter if desired. The filters are then washed to completely remove proteins and other contaminants, and the RNA is eluted in a small volume of Nuclease-free Water or Elution Solution (containing 0.1 mM EDTA to chelate trace amounts of divalent cations). Small cellular RNAs such as tRNA and 5/5.8 S ribosomal RNA are not quantitatively recovered with this kit.

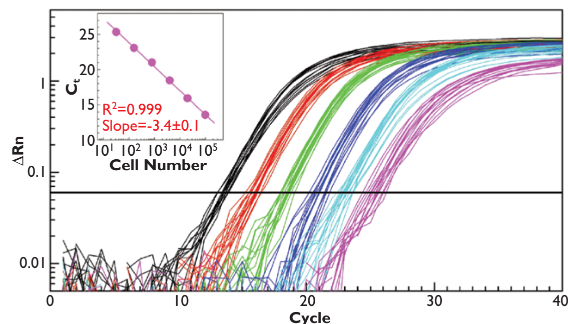

Figure 1. Yield of Total RNA Isolated Using the RNAqueous®-96 Kit.

RNA was extracted from the following quantities of K562 suspension cells: 32, 160, 800, 4,000, 20,000 and 100,000 cells using the RNAqueous-96 Kit. Total RNA yield was ~18 pg/cell. The amount of total RNA recovered was linearly proportional to cell input as quantitated by real-time RT-PCR (5% of the total RNA was used in a 25 µL RT-PCR) using human GAPDH primer/probe set on an ABI 7700.

B. Kit Components and Storage

The RNAqueous-96 Kit provides materials for 192 (2 x 96) RNA purifications.

Amount	Component	Storage
50 mL	Nuclease-free Water	any temp*
50 mL	Elution Solution (0.1 mM EDTA in nuclease-free water)	any temp*
2	Filter Plates	room temp
2	Collection Plates	room temp
2	Adhesive Plate Sealers	room temp
2	Plate Lid	room temp
50 mL	Lysis/Binding Solution‡	4°C
175 mL	Wash Solution Concentrate (Add 140 mL 100% ethanol before use)	4°C†
53 mL	Rebinding Mix‡ (Add 18 mL 100% ethanol before use)	4°C†
12 mL	DNase I Buffer	–20°C
1.25 mL	DNase I (20 units/µL)	–20°C

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

† Components 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.

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

C. Required Materials Not Provided with the Kit

Reagents:

100% ethanol, ACS Grade

Equipment and Supplies:

Vacuum pump or in-house vacuum line and vacuum manifold (see section I. on page 4)

(optional) Centrifuge with rotor and adaptors to hold 96-well plate, and collection plate assembly capable of ~2,000 x g RCF (see section I.F on page 4)

Pipettors and tips or other equipment for liquid transfer

D. Related Products Available from Applied Biosystems

Product	Description
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.
RETROscript® Kit P/N AM1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™, this kit provides reagents, controls and procedures for reverse transcription and PCR. Both oligo(dT) and random primers for cDNA priming are included, as is RNase inhibitor.
NorthernMax® Kits P/N AM1940, AM1946	Ambion NorthernMax Kits: NorthernMax, and NorthernMax-Gly, combine ultrasensitive, reliable Northern blot protocols with unsurpassed quality control to ensure optimal results in less time.

E. Vacuum vs. Centrifugation for Passing Liquid through the Filter Plate

Either vacuum suction and/or centrifugation can be used to pass the sample lysate, wash solutions, and elution solution through the RNAqueous-96 filters. In general, centrifugation is more thorough than vacuum filtration. Centrifugation, however, may require a higher capital investment for equipment. If equipment for both centrifugation and vacuum filtration is available, the best approach is the following:

For section II.D steps:	Preferred method
2.b. Pass the lysate/ethanol through the filter	vacuum
3. (optional) DNase treatment	–
4. Rebind with Rebinding Mix	vacuum
5. Wash filters with Wash Solution (twice)	vacuum
6. Remove residual Wash Solution	centrifuge
7. Elute the RNA	centrifuge

F. Equipment for Centrifuge and Vacuum Manifolds

Equipment

To pass the solutions through the filter plate by centrifugation, a swinging bucket rotor with an adaptor for standard 96-well plates is required. The rotors and adapters listed below can be used in Beckman's Allegra 6 or Spinchron DLX centrifuges:

Rotor	Adapter	Maximum RCF
Beckman GH-3.8	MicroPlus Carrier (part #BK362394)	3200 rpm = 1924 x g
Beckman PTS 2000	Microplate Cannister (part #BK361301)	3200 rpm = 1924 x g

Centrifugation speed

Unless problems with filters rupturing are seen (see section III.B on page 10), centrifugation should be done at 1500 x g (~2000 rpm using the rotors listed above).

Vacuum manifolds

Our filter plates have standard SBS (Society of Biomolecular Screening) footprints, and are compatible with most vacuum manifolds. We recommend using a vacuum manifold with an integrated vacuum gauge and bleed valve.

II. RNAqueous-96 Procedure

A. Sample Size for RNA Isolation

The RNAqueous-96 RNA isolation procedure is designed for processing relatively small samples, i.e. 100–10^6 cells, or 0.1–1.5 mg tissue. Best results are usually obtained using ≤0.5 x 10^6 cells or <1 mg of tissue per well. Sample sizes up to 2 x 10^6 cells per well have been used successfully by passing the lysate through a single well in successive aliquots. Using larger samples increases RNA yield, but the increase may not scale linearly with the amount of sample used. For example, it may be possible to process as much a 2–3 mg of some types of tissue, but the yields may not be proportionally higher compared to using only 1 mg. At some point, yields will actually be reduced by using too much sample, because of interference with RNA binding to the filter by other cellular components.

The best approach to optimize sample size for a particular type of material may be to do a pilot experiment using increasing sample amounts in order to determine the point at which filtration times are prolonged unacceptably, and the yield and/or purity of RNA is diminished. The next few paragraphs discuss some of the parameters that affect optimal sample size for RNAqueous-96.

Purpose of isolating RNA

The upper size limit for samples is somewhat dependent on the intended use of the RNA. For example, when the RNA will be used for hybridization experiments or for poly(A) selection, relatively large samples can usually be used. To avoid trace DNA contamination in RNA that will be used in RT-PCR, on the other hand, relatively small samples should be used.

Sample source

The sample source also influences the amount of lysate that can be processed without clogging the RNAqueous-96 filters. In general, cultured cells are easier to process than solid tissue, because their lysate is less likely to clog the filters even when close to 10^6 cells are used. Some types of tissue are more problematic than others, for example brain and liver are relatively easily filtered but kidney, skeletal muscle, and spleen more often cause filter clogging.

Will vacuum or centrifugation be used for the procedure

Another consideration in determining the upper limit of sample size is the method used to pass the solutions through the filters. Centrifugation usually provides a more powerful force than vacuum filtration, and some vacuum pumps are able to pull a stronger vacuum than others. In general, the greater the force available to move the solutions through the filter, the larger the sample size that can be processed through a single filter well.

B. Before Using the Kit for the First Time

1. Add 18 mL 100% ethanol to the Rebinding Mix

Add 18 mL 100% ethanol (ACS Grade) to the bottle labeled Rebinding Mix. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

2. Add 140 mL 100% ethanol to Wash Solution Concentrate

Add 140 mL 100% ethanol (ACS Grade) to the bottle labeled Wash Solution Concentrate. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

C. Sample Collection and Preparation

As discussed in section *II.A. Sample Size for RNA Isolation* on page 5, a wide range of sample sizes may be used with the RNAqueous-96 Kit, and optimal size depends on several factors. In general, best results will be obtained with the following samples sizes:

Cells in culture: 100 to 0.5×10^6 cells

Tissue samples: 0.1 to 1.0 mg tissue

1a. Cells in culture: remove the cell culture medium

Once the culture medium has been removed, cells should be kept on ice at all times.

It is generally not necessary to wash cells with phosphate buffered saline (PBS) before lysis unless filter clogging has been a problem. If this is the case, wash cells by resuspending in cold PBS and collect them by centrifugation before lysis to help alleviate the problem.

- Cells grown in suspension:
  Count or estimate the number of cells, then pellet them by low speed centrifugation, and thoroughly remove the culture medium.
- Adherent cells:
  Cells may be lysed directly in Lysis/Binding Solution on the surface of the culture vessel after thoroughly removing all culture medium. Tilt culture vessel for a few moments, and remove all the culture medium before adding Lysis/Binding Solution.

  Alternatively, cells may be released from the surface of the culture flask by trypsinization or scraping, then pelleted by centrifugation. Count or estimate the number of cells.

1b. Solid tissue samples

If the source organism will be sacrificed, tissue should be obtained as quickly as possible after sacrifice. Immediately after obtaining the tissue, it must be either processed in Lysis/Binding Solution, or placed into storage. We recommended Ambion RNA*later*® Solution for both short- and long-term storage of tissue samples because, unlike tissue that is stored by freezing in liquid nitrogen, RNA*later*-stored tissue usually does not need to be ground to a powder in liquid nitrogen for RNA isolation.

2. Disrupt sample in Lysis/Binding Solution

a. Cells in culture: add 100–200 μL Lysis/Binding Solution, and vortex or pipette the cells to disrupt them.

The exact volume of Lysis/Binding Solution is not critical, but in general, low-end amounts of Lysis/Binding Solution should be used for fewer cells (10–10,000) and high-end amounts should be used for more cells (>10,000–1,000,000).

For >1,000,000 cells, use more Lysis/Binding Solution (up to 600 μL per 2,000,000 cells). The kit provides enough Lysis/Binding Solution for a maximum of 250 μL per sample for 192 samples; if more Lysis/Binding Solution is needed it can be purchased separately from Ambion. The more cells used, the longer the sample will need to be vortexed for complete disruption.

b. Solid tissue: homogenize in 100–250 μL Lysis/Binding Solution.

NOTE

Comprehensive information on tissue disruption can be found on www.Ambion.com – click Technical Resources, then choose The Basics/General Articles/RNA Isolation).

Small samples, like fine-needle aspirates or tissue punches, should generally be disrupted in about 200 μL of Lysis/Binding Solution. Very small tissue samples may be disrupted in less Lysis/Binding Solution, and conversely, tissue samples larger than ~1 mg may require more Lysis/Binding Solution (up to 600 μL for up to 2 mg).

D. RNA Isolation from Sample Lysate

1. Mix the lysate with 1/2 volume of 100% ethanol

Add a volume of 100% ethanol equal to one half the volume of Lysis/Binding Solution used in step II.C.2 to the lysate and mix briefly by repeated pipetting, or gently shaking.

2. Pass the mixture through the wells of the Filter Plate

a. Cover unused wells

Wells which are not used must be blocked by covering them with an Adhesive Plate Sealer (provided). An easy way to do this is to cover the entire plate with the film, then use a scalpel or razor blade to cut the film away from the wells that will be used. Carefully peel away and discard the unwanted portions of the film. Be sure the Plate Sealer is pressed down securely around unused portions of the plate.

b. Pass the lysate/ethanol through the filter

For the sake of convenience, the recommended method for filtering the lysate is by vacuum. The time required to pull the lysate through the filter by vacuum filtration will vary; several minutes are required in most cases.

If centrifugation is used to draw the lysate through the Filter Plate, use a receptacle with enough capacity to catch the flow-through. Typical centrifugation times are 1–5 min.

3. (optional) DNase treatment

For applications such as amplification of mRNA targets by RT-PCR, it may be desirable to treat the RNA with DNase to remove contaminating genomic DNA. DNase treatment is generally recommended when PCR does not distinguish between products amplified from DNA vs. RNA templates. DNase treatment may not be necessary when the sample size is small, for example fewer than 5,000 cells, or less than ~0.2 mg of tissue per well. If the sample is known to be rich in lipids and/or proteins, include an extra wash before starting the procedure as described in section *III.F. Protein/lipid rich samples may require an additional wash step before the DNase treatment* on page 12.

a. Wash filters with 300 μL per well of Wash Solution. Pass the solution through the filters by vacuum or centrifugation.

b. Remove residual Wash Solution from filters by applying vacuum pressure for an extra 3 min, or by centrifuging for 2 min after the Wash Solution has passed through.

c. Prepare a DNase I working solution by ***gently*** mixing 2.5 μL of the DNase I with 17.5 μL of DNase I Buffer per sample. ***Do not vigorously vortex the DNase solution.*** It is a good idea to prepare about 10% extra DNase I working solution to compensate for pipetting error (kit provides sufficient reagents to allow for this). For example, to make DNase I working solution for 20 samples, mix:

   2.5 μL X 20 samples = 50 μL + 10% = 55 μL DNase 1
   17.5 μL X 20 samples = 350 μL + 10% = 385 μL DNase 1 Buffer

d. Add 20 μL of DNase I working solution to the center of each filter pad, and incubate the plate for 20 min at room temp. For larger samples (>100,000 cells or >1 mg tissue), the incubation may be done at 37° for 30 min to increase the efficiency of DNA degradation.

4. Add 240 μL per well of Rebinding Mix

Add 240 μL of Rebinding Mix per well to rebind RNA. Pass the solution through the filters by vacuum or centrifugation.

5. Wash filters with 300 μL Wash Solution

Wash filters with 300 μL per well of Wash Solution. Pass the solution through the filters by vacuum or centrifugation.

6. Wash filters with 200 μL Wash Solution

Wash filters with 200 μL per well of Wash Solution. Pass the solution through the filters by vacuum. To thoroughly remove all Wash Solution from filters, vacuum for an extra 3 min, or centrifuge (recommended) for 2 min after Wash Solution has passed through.

7. Elute the RNA with ≥50 μL Elution Solution

Total RNA can be eluted from the Filter Plate with either Nuclease-free Water or Elution Solution. Ambion recommends using Elution Solution (0.1 mM EDTA) for long term RNA storage, and Nuclease-free Water when the RNA will be used soon after isolation and/or in downstream applications that cannot tolerate 0.1 mM EDTA.

a. Place a clean 96-well Collection Plate (from the kit) under the filter plate.

b. Apply desired amount (≥50 µL) of Elution Solution, or Nuclease-free Water, to the center of each well. The exact volume of elution is not critical and may be adjusted according to sample size and user preference, but 50 µL is the minimum volume recommended for efficient recovery of the RNA.

c. Pass the solution through the filter by centrifuging at ~1500 x g for 2 min (recommended method) or by applying vacuum for ~2 min.

8. Repeat the RNA elution with a second ~30–60 µL of Elution Solution

The eluted RNA may be stored in the Collection Plate, covered first with an Adhesive Plate Sealer and then with a Plate Lid. Using both the Adhesive Plate Sealer (placed directly over the open wells) and the Plate lid (fitted over the Adhesive Plate Sealer) will make a tight seal.

Store at –20°C for short-term storage and –80°C for long-term storage.

III. Troubleshooting

A. Filter Plate Clogs

Too much sample was applied and/or the lysate is too viscous

Reduce the viscosity of the lysate by either using more Lysis/Binding Solution to homogenize the sample, and/or by using smaller samples (fewer cells or smaller piece of tissue). Viscosity can also be reduced by shearing the DNA. This can be done by passing the sample through a syringe needle (20–25 gauge) several times, or by sonicating the lysate.

More force is needed to pull the lysate through the filter

Increase the force used to move the solutions through the filters

- Use stronger vacuum suction. Depending on your vacuum setup, this can be done by using a stronger vacuum pump, using a tighter manifold, or by simply using fewer wells of the filter plate at one time
- Use centrifugation instead of vacuum filtration
- Centrifuge at a higher RCF

B. Filters Rupture during Centrifugation

Filters occasionally rupture when processing lysate volumes that exceed the capacity of a single well, and must therefore be passed through the filter in multiple aliquots. Filters may also rupture, and/or plates crack when centrifuged at high speeds or for prolonged times.

Use milder centrifugation

If RNAqueous-96 filters rupture, reduce the centrifuge speed for drawing lysates through the filters and for the wash steps, and reduce the centrifugation time, and/or speed used for removing Wash Solution and for RNA elution. For example, centrifuge at 200 x g for ~4 min for the lysate filtration and wash steps.

C. Plates Crack during Centrifugation

Make sure that the plates are well seated in the adapter

Because they are made of polystyrene, the RNAqueous-96 Filter and Collection Plates are somewhat brittle. During centrifugation, it is important that the plates are properly supported by the adapter so that they can withstand the centrifugal force.

D. DNA Contamination Remains after DNase Treatment

Residual Wash Solution on the filter

Be sure to completely remove Wash Solution before the DNase treatment. The presence of residual Wash Solution may decrease the enzyme's activity.

Use less sample

Reducing the number of cells or the mass amount of tissue processed per well will increase the efficiency of DNase treatment. RNA isolated from some tissues such as spleen, thymus, and kidney, is more prone to having DNA contamination and with these samples, smaller sample sizes must be used to avoid DNA contamination.

Design PCR primers to span one or more intron/exon boundaries

If contaminating DNA is suspected because PCR product is made from a minus-RT control in RT-PCR, using PCR primers that span an intron/exon boundary will minimize the effect of the contaminating DNA. Primers that span multiple introns or long introns, will typically span a region of genomic DNA that would be too large for amplification under the conditions used to amplify the desired shorter RT-PCR product. Primers spanning short introns generally yield a product from contaminating DNA that is distinguishable by size from the RT-PCR product amplified from RNA.

Do a post-elution DNase treatment

The DNase I and DNase I Buffer provided with the RNAqueous-96 Kit are optimized for on-the-filter digestion, and may not be ideal for treatment of RNA in solution. A better option for post-elution DNase treatment is to use the Ambion DNA-*free*™ kit (P/N AM1906), which provides reagents for DNase digestion and subsequent removal of DNase and buffer from the sample.

E. Lower-than-Expected RNA Yield

Be sure the expectations for RNA yield are realistic for the type of sample used: some tissues, for example brain and skeletal muscle, have relatively low RNA content.

The filters were overloaded

Using too much starting sample may reduce the RNA binding efficiency of the RNAqueous-96 filters. The maximum amount of starting material that can be used without compromising RNA yield and quality depends on the type of sample (cultured cells vs. tissue samples, and tissue source), and may need to be determined experimentally for particular samples. See section II.A on page 5 for more information on sample size.

Elute RNA with hot Elution Solution

RNA yields may be increased by eluting in heated (≤100°C) Elution Solution, or Nuclease-free Water.

Elute the RNA several times

More RNA may be obtained in some cases by including additional elution steps; this is usually more effective when larger sample sizes have been used.

F. RNA Is Degraded

Protein/lipid rich samples may require an additional wash step before the DNase treatment

With some protein/lipid rich samples, cellular nucleases may be immobilized on the filter with the RNA. Thus, some RNA degradation may occur during the DNA digestion step. If this is the case, add an additional wash before the DNase treatment with the modified wash solution described below.

Pass 200 µL of modified wash solution through the filter from step II.D.2.b on page 7 by vacuum pressure or centrifugation, and then proceed to step II.D.3.a.

Modified wash solution:
2:1 mixture of Lysis/Binding Solution and 100% ethanol (extra Lysis/Binding Solution for this application can be purchased separately from Applied Biosystems).

Run a positive control on gels to rule out gel artifacts

When assessing RNA intactness by electrophoresis on denaturing agarose gels, always run a positive control RNA sample known to be intact as a control for electrophoresis conditions and for degradation introduced during gel analysis.

Improper handling of samples before RNA isolation

Most problems with RNA degradation are caused by improper storage or handling of samples before lysis/disruption. Solid tissue must be obtained quickly, and either homogenized in Lysis/Binding Solution, placed into RNA*later* storage solution, or snap-frozen in liquid nitrogen to prevent degradation of the RNA by intracellular RNases.

Contaminated RNAqueous-96 kit components or pipette tips

A less likely cause of RNA degradation is due to contaminating RNases introduced by pipette tips or mishandling of the RNAqueous-96 kit reagents. To avoid contaminating the kit reagents, take precautionary measures such as using RNase-free pipette tips, wearing gloves when opening tubes and bottles, and keeping the vessels closed when not in use. Ambion RNase*Zap*® Solution (P/N AM9780–AM9784) can be used to eliminate RNases from the lab bench and from other equipment such as pipettors.

G. Variation in RNA Yield between Wells

Uneven vacuum suction

Uneven vacuum suction during the filtration and elution steps can cause variation in RNA yield between wells of the RNAqueous-96 Filter Plate. To improve uniformity of vacuum pressure across the plate, be sure to cover unused wells completely, and/or use a device such as the Vacuum Assist Frame (included with the Whatman/Polyfiltronics vacuum manifold) to press down on the filter plate when the vacuum is applied.

Other suggestions

- For filtration by centrifugation, be sure the plate carrier swings freely during centrifugation.
- Also, well to well reproducibility requires accurate delivery of uniform volumes of Elution Solution to each well.
- Be sure to elute RNA in at least 50 µL Elution Solution, or Nuclease-free Water, and apply it directly to the center of the filters for the best results.

IV. Additional Procedures

A. Quantitation and Assessment of RNA Purity by UV Absorbance

The concentration and purity of RNA can be determined by diluting an aliquot of the preparation (usually a 1:0 to 1:50 dilution. If NanoDrop is used, no dilution is necessary) in TE (10 mM Tris-HCl pH 8, 1 mM EDTA), and reading the absorbance in a spectrophotometer at 260 nm and 280 nm. Be sure to zero the spectrophotometer with the TE used for sample dilution.

1. Concentration

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

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

Following is a typical example:

RNA is eluted in 100 µL Nuclease-free Water
10 µL of the prep is diluted 1:10 into 100 µL of TE
A_{260} = 0.25
RNA concentration = 0.25 X 10 X 40 µg/mL = 100 µg/mL or 0.1 µg/µL
Since there are only 90 µL of the prep left after sacrificing 10 µL to measure the concentration, the total amount of remaining RNA is:
90 µL X 0.10 µg/µL = 9.0 µg

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.

2. Purity

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

B. Assessing RNA Integrity with Agilent Bioanalyzer

Agilent's 2100 bioanalyzer used in conjunction with one of the RNA LabChip® Kits provide a powerful and sensitive method to assess RNA integrity. To use this system, follow the instructions for RNA analysis provided with the RNA LabChip Kit.

The 28S/18S rRNA ratio is typically used as an indicator of RNA integrity. RNA isolated with this kit should have a 28S/18S rRNA ratio of 1.5–2.0.

C. Assessing RNA on Denaturing Agarose Gel

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.

The Ambion NorthernMax® (P/N AM1940, AM1946) reagents include everything needed for denaturing agarose gel electrophoresis.

Alternatively, use the procedure described below. This method for RNA electrophoresis 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 involving formaldehyde should be done in a chemical fume hood.

10X MOPS running buffer

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

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

b. 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. In a nuclease-free tube set up the RNA denaturation mix.

RNA denaturation mix

2 μL	RNA (1–3 μg)
2 μL	10X MOPS buffer
4 μL	formaldehyde
10 μL	formamide
1 μL	ethidium bromide (200 μg/mL)

NOTE

RNA size markers should be prepared in the same way as the sample RNA.

a. Incubate the RNA denaturation mix for 10 min at 85°C in a closed microfuge tube.

b. Transfer the samples to ice and allow to cool for 10 min.

c. Add 2 µL of 10X formaldehyde gel-loading buffer to each sample and return the tubes to an ice bucket.

10X formaldehyde gel-loading buffer

50 %	Glycerol (diluted in nuclease-free water)
10 mM	EDTA (pH 8.0)
0.25 %	(w/v) bromophenol blue
0.25 %	(w/v) xylene cyanol FF

3. Electrophorese

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. Visualize the RNA on a UV transilluminator.

4. Results

A typical denaturing agarose gel containing RNA isolated with RNAqueous-96 is shown in Figure 2. The 28S and 18S ribosomal RNA bands should appear as sharp, intense bands (size is dependent on the organism from which the RNA was obtained). A diffuse smear of ethidium bromide-stained material will normally be seen migrating between the ribosomal bands, this is mRNA and other heterogeneous RNA species. Very small RNA species (e.g. 5S rRNA and tRNA) will not be efficiently recovered with the RNAqueous-96 Kit.

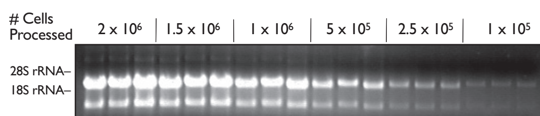

Figure 2. RNA from K562 Leukemic Cells Isolated Using the RNAqueous®-96 Kit

K562 cells were lysed at a concentration of 3.3×10^6 cells/mL, and aliquots containing the indicated number of cells were processed in triplicate with the RNAqueous-96 Kit. The RNA (10% of each prep) was analyzed on an ethidium bromide-stained, denaturing agarose gel.

5. Potential problems

It is important not to overload the gel. If more than about 5 µg of total RNA is run in a single lane, resolution of the ribosomal RNA bands will be poor, and it will be difficult to assess the quality of the RNA. Incomplete denaturation of the samples may also cause smearing. The RNA should be considered degraded only if the gel contained a lane with a positive control that looks intact.

V. Appendix

A. References

Chirgwin, J., Przybyla, A., MacDonald, A., and Rutter, W. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem.* **18**:5294.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**:156–159.

B. Quality Control

Functional testing

All kit lots are tested functionally by isolating RNA from cells using the procedure described in this protocol and subjecting the RNA to RT-PCR.

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.

Protease testing

Meets or exceeds specification when a sample is incubated with protease substrate and analyzed by fluorescence.

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.