MagMAX™-96 Total RNA Isolation Kit

(Cat #AM1830)

Instruction Manual

Manual 1830M Revision B **Revision Date: July 31, 2007**

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I. Introduction

A. Product Description

The MagMAX™-96 Total RNA Isolation Kit is designed for rapid high throughput purification of total RNA in 96-well plates. The kit is optimized for use with either manual multichannel pipettors or with robotic liquid handlers. The microspherical magnetic beads used in this procedure have a large available binding surface and can be fully dispersed in solution, allowing thorough RNA binding, washing, and elution. The procedure, therefore, delivers high quality RNA with very consistent yield.

High yield of total RNA can be obtained with the MagMAX-96 Total RNA Isolation Kit from the following sample types and quantities:

- 25 to 2 x 10^6 cultured mammalian cells—RNA yield is typically 5–20 pg RNA per cell, depending on the cell type and growth conditions.
- ≤5 mg animal tissue
- ≤5–10 mg plant tissue

The MagMAX-96 Total RNA Isolation Kit can also be used for isolation of viral RNA from these sample types. For viral RNA isolation from biological fluids, such as serum, plasma, and swabs, we recommend using the MagMAX-96 Viral RNA Isolation Kit (Cat #AM1835).

B. Overview of the Procedure

The MagMAX-96 Total RNA Isolation Kit employs a classic method for disrupting samples in a guanidinium thiocyanate-based solution that rapidly solubilizes cellular membranes and simultaneously inactivates nucleases (Chirgwin et al., 1979; Chomczynski and Sacchi 1987). Optimized protocols and disruption/lysis formulations are provided to achieve maximum RNA yield from cultured mammalian cells, animal tissues, and plant tissues. After homogenization, samples are mixed with magnetic beads that have a nucleic acid binding surface. The beads and bound nucleic acids are then magnetically captured and washed to remove cell debris, protein, and other contaminants. Next, the nucleic acid is treated with DNase and is purified from the reaction mixture. Finally, RNA is eluted in 50 µL of low salt buffer (Figure 1).

Figure 1. MagMAX™-96 Total RNA Isolation Procedure

Sample Homogenization

Cultured mammalian cells

1. Prepare Lysis/Binding Solution
2. Lyse up to 2 x 10^6 cells in 140 µL prepared Lysis/Binding Solution
3. Shake for 1 min

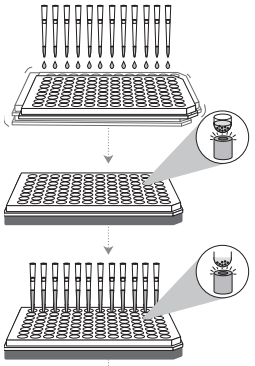
Animal tissue samples

1. Prepare Lysis/Binding Solution
2. Homogenize up to 5 mg of tissue in 100 µL prepared Lysis/Binding Solution
3. Add 60 µL100% isopropanol and shake for 1 min

Plant tissue samples

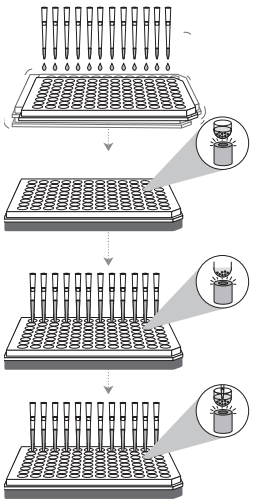
1. Prepare Lysis/Binding Solution
2. Homogenize up to 10 mg tissue in 100 µL prepared Lysis/Binding Solution
3. Centrifuge lysate at 1000 x g for 10 min at room temp
4. Transfer 50 µL of lysate to the Processing Plate, add 35 µL100% isopropanol, and shake for 1 min

Initial Nucleic Acid Purification



1. Add 20 µL Bead Mix to each sample; shake for 5 min
2. Magnetically capture the RNA Binding Beads and discard the supernatant
3. Wash with 150 µL Wash Solution 1 for 1 min with shaking
4. Wash with 150 µL Wash Solution 2, and prepare Diluted TURBO™ DNase

TURBO DNase™ Treatment and Final RNA Clean-Up



1. Add 50 µL of Diluted TURBO DNase and shake for 10–15 min at room temp
2. Add 100 µL RNA Rebinding Solution and shake for 3 min to rebind the RNA
3. Magnetically capture the RNA Binding Beads and discard the supernatant
4. Wash twice with 150 µL Wash Solution 2 each time
5. Dry the beads by shaking for 2 min
6. Elute the RNA in 50 µL of Elution Buffer

RNA suitable for most downstream applications

The quality and purity of the eluted RNA make it suitable for most common downstream application, such as qRT-PCR, microarray analysis, Northern blotting, and ribonuclease protection assays (RPA). Figure 2 shows an example of qRT-PCR data that illustrates linear RNA recovery from a broad range of input cell amounts using the MagMAX-96 Total RNA Isolation Kit.

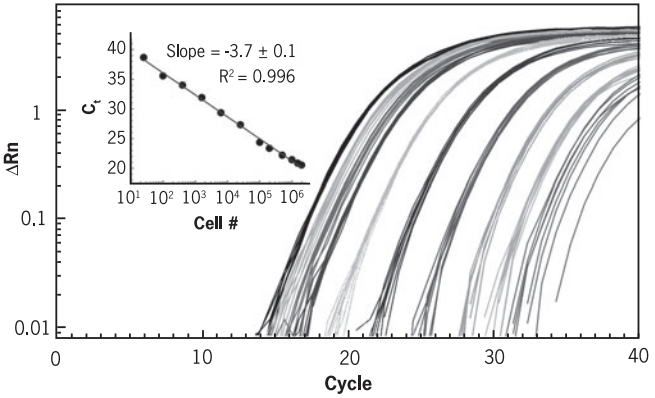


Figure 2. Linear Recovery of RNA Using the MagMAX™-96 Total RNA Isolation Kit

RNA was isolated from K562 cells in 8 replicate wells using the MagMAX-96 Total RNA Isolation Kit. Equivalent volumes (4% of eluent) of the recovered RNA were then used in real-time qRT-PCR targeting the human RNA Polymerase II gene (RPII). The CV of C_t values among replicates was less than 3%. The lines represent amplification products from RNA obtained from the following sample sizes (left to right): 2 x 10^6, 10^6, 2 x 10^5, 10^5, 2.5 x 10^4, 6.25 x 10^4, 1600, 400, 100, and 25 cells.

C. Extending Utility of the Kit

Recover total nucleic acids

The MagMAX-96 Total RNA Isolation Kit is designed for RNA isolation; however, by omitting the DNase treatment from the protocol, it can also be used for total nucleic acid isolation. To isolate both RNA and DNA with the kit, omit steps III.A.4 through III.B.3 starting on page 13, but otherwise follow the protocol for RNA isolation.

Note that excessive amounts of DNA can cause the RNA Binding Beads to clump together, which may result in inefficient elution of DNA/RNA at the end of the procedure. If you experience low yield and/or inconsistent yield, lowering the amount of sample input may improve results.

More information on Ambion's high throughput technologies

We are continually working to improve our technologies and expand our line of high throughput products. High throughput applications may require customized solutions. For detailed information regarding your specific application, contact us at:

highthroughput@ambion.com

D. Kit Components and Storage Conditions

The MagMAX-96 Total RNA Isolation Kit contains reagents to isolate RNA from 96 samples.

Amount	Component	Storage
1	Processing Plate with Lid	room temp
11 mL	Lysis/Binding Solution Concentrate (See section II.B starting on page 9 for instructions)	room temp
18 mL	Wash Solution 1 Concentrate (Add 6 mL 100% isopropanol before use)	room temp
55 mL	Wash Solution 2 Concentrate (Add 44 mL 100% ethanol before use)	room temp
12 mL	RNA Rebinding Concentrate (Add 6 mL 100% isopropanol before use)	room temp
10 mL	Elution Buffer	4°C or room temp
6 mL	MagMAX™ TURBO™ DNase Buffer	4°C or room temp
1.1 mL	RNA Binding Beads	4°C*
1.1 mL	Lysis/Binding Enhancer	–20°C
110 µL	TURBO™ DNase	–20°C

* Do not freeze the RNA Binding Beads.

E. Required Materials Not Provided With the Kit

Reagents/equipment

- 100% ethanol, ACS grade or higher quality
- 100% isopropanol, ACS grade or higher quality
- To use the kit manually, you will need an orbital shaker for 96-well plates such as the Barnstead/Lab-Line Titer Plate Shaker (available from VWR and Fisher Scientific).
- Magnetic stand for 96-well plates: We recommend either of the Ambion 96-well magnetic stands (Cat #AM10050, AM10027) for their high strength magnets and quality design (See *I.F. Related Products Available from Ambion* on page 6).
- If you process fewer than 96 samples at a time, you will need additional polystyrene U bottom 96-well plates and lids.

RNA isolation from animal tissue samples

- 14.3 M β-mercaptoethanol (β-ME)

RNA isolation from plant tissue samples

- Plant Isolation Aid (Ambion Cat #AM9690)

Automation equipment

For completely automated RNA isolation with the MagMAX-96 Total RNA Isolation Kit, the robotic liquid handler must have the following features:

- 200 µL pipetting tool
- Gripper tool
- Six reservoirs with reservoir holders
- One magnetic stand for 96-well plates
- Integrated orbital shaker (e.g., MicroMix-5 from Diagnostic Products Corporation).

F. Related Products Available from Ambion

96-well Magnetic-Ring Stand Cat #AM10050	The Ambion 96-well Magnetic-Ring Stand features 96 powerful ring-shaped magnets arranged to cradle each well of a 96-well plate for quick, thorough bead capture. Captured magnetic beads form evenly distributed donut-shaped pellets with a large hole in the center. This capture pattern facilitates both supernatant removal and subsequent bead resuspension. The stand is suitable for high throughput applications conducted with multi-channel pipettors or with robotic liquid handlers. However, because the pellets will be evenly distributed around the edge of the wells, it may require practice for efficient manual removal of supernatants.
Magnetic Stand-96 Cat #AM10027	The Ambion Magnetic Stand-96 has powerful magnets positioned to capture beads to one side of the well. This capture pattern makes it very easy to remove supernatants manually without disturbing the beads, and therefore may be preferred by beginning users. In some applications, however, pellets formed with the Magnetic Stand-96 may be difficult to resuspend. If this occurs, we recommend the 96-well Magnetic-Ring Stand (Cat #AM10050).
Plant RNA Isolation Aid Cat #AM9690	The Plant RNA Isolation Aid contains polyvinylpyrrolidone (PVP) to selectively remove polysaccharides and polyphenolics from plant lysates during RNA isolation. It is compatible with most RNA isolation procedures that use chaotropic salt-based lysis solutions.
RNA*later*® Solution Cat #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 #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 #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.

MessageAmp™ II aRNA Amplification Kits see our web or print catalog	Ambion offers a full line of MessageAmp II Kits tailored for different array analysis applications. The MessageAmp II Kit offers maximum flexibility; samples can be amplified using either single- or double-round amplification, and the reagent cocktails are configured to accommodate modification. For arrays requiring biotin-labeled samples, Ambion offers the MessageAmp II-Biotin *Enhanced* Single Round aRNA Amplification Kit. For preparation of fluorescently-labeled samples, we recommend the Amino Allyl MessageAmp II Kits which are available with and without Cy™3 and Cy5. Bacterial RNA can be amplified using the MessageAmp II Bacteria RNA Amplification Kit. We also offer the MessageAmp II-96 and Amino Allyl MessageAmp II-96 aRNA Amplification Kits for high throughput applications.
MessageSensor™ RT Kit Cat #AM1745	The MessageSensor RT Kit for one-step qRT-PCR includes an optimized set of reagents for exceptionally sensitive reverse transcription. The kit is designed to be used for single-tube amplification of mRNA using either real-time or end-point amplification strategies.

II. Preparation and Sample Homogenization

A. Equipment and Reagent Preparation

1. RNase precautions

Lab bench and pipettors

Before working with RNA, it is always a good idea to clean the lab bench and pipettors with an RNase decontamination solution (e.g., Ambion RNase*Zap*® Solution, Cat #AM9780).

Gloves and RNase-free technique

Wear laboratory gloves for this procedure; they protect you from the reagents, and they protect the RNA from nucleases that are present on skin.

Use RNase-free pipette tips to handle the kit reagents, and avoid putting used tips into the reagent containers.

2. Determine maximum 96-well plate shaker settings

For larger volumes

Place 200 µL water in the wells of a 96-well plate and use it to determine the maximum shaker setting that can be used with your orbital shaker without sample spillage. This maximum shaker speed will be used for most steps of the procedure.

For smaller volumes

Place 50 µL of water in the wells of a 96-well plate and use it to determine the maximum shaker setting that can be used with your orbital shaker without sample spillage. Use this speed for the bead drying and RNA elution steps (steps III.B.5 and 6 on page 15 respectively).

3. Before using the kit, complete Wash Solutions 1 and 2, and RNA Rebinding Solution

a. Add 6 mL 100% isopropanol to the bottle labeled Wash Solution 1 Concentrate and mix well.

   The resulting mixture is called Wash Solution 1 in these instructions.

b. Add 44 mL 100% ethanol to the bottle labeled Wash Solution 2 Concentrate and mix well.

   The resulting mixture is called Wash Solution 2 in these instructions.

c. Add 6 mL 100% isopropanol to the bottle labeled RNA Rebinding Concentrate and mix well.

   The resulting mixture is called RNA Rebinding Solution in these instructions.

d. Mark the labels of the solutions to indicate that isopropanol or ethanol was added. Store the solutions at room temperature.

4. Prepare Bead Mix

Each isolation reaction requires 20 µL of Bead Mix. Although the mixture is tested to be stable at 4°C for up to 2 weeks, we recommend preparing Bead Mix on the day it will be used.

a. Vortex the RNA Binding Beads at moderate speed to form a uniform suspension before pipetting.

b. Prepare Bead Mix by combining the volumes of RNA Binding Beads and Lysis/Binding Enhancer shown in Table 1 below appropriate for the number of isolation reactions to be performed that day. Mix thoroughly.

We recommend including ~10% overage to cover pipetting error when preparing the Bead Mix.

Table 1. Bead Mix preparation

Component	Per reaction	96 rxns (+10%)
RNA Binding Beads	10 µL	1.1 mL
Lysis/Binding Enhancer	10 µL	1.1 mL

c. Place the prepared Bead Mix on ice until it is needed in step III.A.1.

B. Sample Homogenization

Sample homogenization instructions optimized for different sample types are listed below. Follow the instructions in this section for cultured mammalian cell (section B.I below), animal tissue samples (section B.II on page 10), or plant tissue samples (section B.III on page 11).

B.I. Cultured Mammalian Cells

1. Prepare Lysis/Binding Solution

- We recommend including 5–10% overage to cover pipetting error.
- Store the Lysis/Binding Solution for cultured cells at room temperature, do not store at 4°C. If the prepared Lysis/Binding Solution is inadvertently stored at 4°C, warm it at room temperature and vortex to dissolve any precipitate that forms—do not heat it.

Table 2. Lysis/Binding Solution For Cultured Cells

Per reaction	Entire bottle	Reagent
77 µL	11 mL	Lysis Binding Solution Concentrate
63 µL	9 mL	100% isopropanol
140 µL	20 mL	total Lysis/ Binding Solution

- After assembling the Lysis/Binding Solution, mix thoroughly.

2. Lyse up to 2 x 10^6 cells in 140 µL prepared Lysis/Binding Solution

Adherent cells: Remove culture media from 2.5 x 10^2 to 2 x 10^6 cells, and immediately add 140 µL Lysis/Binding Solution.

Suspension cells: Suspend cells in ≤30 µL of 1X PBS or in the Ambion RNA*later*® tissue collection: RNA stabilization solution. Transfer cells to wells of a Processing Plate, and add 140 µL Lysis/Binding Solution.

3. Shake for 1 min

Shake for 1 min on an orbital shaker at the maximum speed for larger volumes identified in step A.2 on page 8. Cells will lyse during this shaking incubation.

Adherent cells: Transfer 140 µL cell lysate to wells of the Processing Plate.

Proceed to step III.A.1 on page 13.

B.II. Animal Tissue Samples

1. Prepare Lysis/Binding Solution

a. Lysis/Binding Solution for processing animal tissue samples must be prepared on the day it will be used. Prepare Lysis/Binding Solution for the number of samples to be processed that day, plus 5–10% overage. Store at room temperature.

Table 3. Lysis/Binding Solution For Animal Tissue Samples

Per reaction	Entire bottle	Reagent
100 µL	11 mL	Lysis Binding Solution Concentrate
0.7 µL	77 µL	β-mercaptoethanol (14.3 M)
~100 µL	~11 mL	total Lysis/ Binding Solution

b. After assembling the Lysis/Binding Solution, mix thoroughly.

2. Homogenize up to 5 mg of tissue in 100 µL prepared Lysis/Binding Solution

Disrupt and homogenize samples in 100 µL prepared Lysis/Binding Solution using standard homogenization procedures. For most tissues, rotor-stator homogenizers work very well.

Sample size: For most animal tissue types, 5 mg samples can be processed per reaction. But for tissues that are very high in nucleases, such as spleen and pancreas, use 2.5 mg or less tissue per sample.

Handling fresh tissue: Immediately after dissection, inactivate RNases by any one of the following treatments:

- Freeze rapidly in liquid nitrogen (tissue pieces must be small enough to freeze in a few seconds).
- Submerge in a tissue storage buffer such as Ambion RNA*later* solution.
- Homogenize in prepared Lysis/Binding Solution immediately.

NOTE

For a detailed discussion tissue disruption for RNA isolation, see Technical Bulletin #183 on our website: www.ambion.com/techlib//tb_183.html

Handling frozen tissue: See section *V.C. Handling Frozen Tissue for RNA Isolation* on page 20 for detailed suggestions. Weigh frozen tissue, and if necessary, break it into 2.5–5 mg pieces (keeping tissue completely frozen) and homogenize directly in a rotor-stator homogenizer. Larger pieces of tissue, very hard or fibrous tissues, and tissues with a high RNase content, are typically ground into powder in liquid nitrogen for maximum RNA yield.

Cleanup and decontamination: Wipe the benchtop and all utensils and containers used for tissue handling with a 10% bleach solution to clean and decontaminate them.

3. Add 60 µL100% isopropanol and shake for 1 min

a. Transfer homogenized sample to wells of the Processing Plate.

b. Add 60 µL 100% isopropanol to each sample.

c. Shake for 1 min on an orbital shaker at the maximum speed for larger volumes identified in step A.2 on page 8.

Proceed to step III.A.1 on page 13.

B.III. Plant Tissue Samples

1. Prepare Lysis/Binding Solution

a. Lysis/Binding Solution for processing plant tissue samples must be prepared on the day it will be used. Prepare Lysis/Binding Solution for the number of samples to be processed that day, plus 5–10% overage. Store at room temperature.

Table 4. Lysis/Binding Solution For Plant Tissue Samples

Per reaction	Entire bottle	Reagent
90 µL	11 mL	Lysis Binding Solution Concentrate
10 µL	1.22 mL	Plant RNA Isolation Aid (Ambion Cat #AM9690)
100 µL	12.22 mL	total Lysis/ Binding Solution

b. After assembling the Lysis/Binding Solution, mix thoroughly.

2. Homogenize up to 10 mg tissue in 100 µL prepared Lysis/Binding Solution

Disrupt and homogenize samples in 100 µL prepared Lysis/Binding Solution using standard homogenization procedures for the plant tissue type. Optimal disruption and homogenization procedures vary widely for different plant tissue types, refer to the recent scientific literature for information on disruption of your sample type.

3. Centrifuge lysate at 1000 x g for 10 min at room temp

Centrifuge homogenized sample at 1000 x g for 10 min at room temp to remove insoluble debris.

4. Transfer 50 µL of lysate to the Processing Plate, add 35 µL100% isopropanol, and shake for 1 min

a. Transfer 50 µL of homogenized sample to wells of the Processing Plate.

b. Add 35 µL 100% isopropanol to each sample.

c. Shake for 1 min on an orbital shaker at the maximum speed for larger volumes identified in step A.2 on page 8.

Proceed to step III.A.1 on page 13.

III. MagMAX-96 Total RNA Isolation Protocol

A. Initial Nucleic Acid Purification

1. Add 20 µL Bead Mix to each sample; shake for 5 min

a. Gently vortex the Bead Mix (prepared in step II.A.4 on page 9) to resuspend the magnetic beads. Add 20 µL Bead Mix to each sample.

b. Shake for 5 min on an orbital shaker at the maximum speed for larger volumes identified in step A.2 on page 8 to bind the RNA to the RNA Binding Beads.

2. Magnetically capture the RNA Binding Beads and discard the supernatant

a. Move the Processing Plate to the magnetic stand to capture the RNA Binding Beads. Leave the plate on the magnetic stand until the beads have completely pelleted. The capture time depends on the magnetic stand used; with the Ambion 96-well Magnetic-Ring Stand, the capture time is ~2–3 min.

b. Carefully aspirate and discard the supernatant without disturbing the beads, and remove the Processing Plate from the magnetic stand.

3. Wash with 150 µL Wash Solution 1 for 1 min with shaking

a. Add 150 µL Wash Solution 1 (isopropanol added) to each sample and shake for 1 min at the maximum speed for larger volumes.

b. Move the Processing Plate to a magnetic stand to capture the RNA Binding Beads. Leave the plate on the magnetic stand until the mixture becomes transparent, indicating that capture is complete. The capture time depends on the magnetic stand used; with the Ambion 96-well Magnetic-Ring Stand, the capture time is ~1–2 min.

c. Carefully aspirate and discard all supernatant without disturbing the beads, and remove the Processing Plate from the magnetic stand.

It is critical to remove the Processing Plate from the magnetic stand before the subsequent wash step.

4. Wash with 150 µL Wash Solution 2, and prepare Diluted TURBO™ DNase

a. Add 150 µL Wash Solution 2 (ethanol added) to each sample and shake for 1 min on an orbital shaker at the maximum speed for larger volumes.

b. Capture the RNA Binding Beads on a magnetic stand. During this capture step, prepare the diluted TURBO™ DNase as described in the next step (4.c).

c. While capturing the RNA Binding Beads, combine the volumes of MagMAX TURBO DNase Buffer with TURBO DNase shown in the table below appropriate for the number of samples being processed plus ~10% overage to cover pipetting error. Mix thoroughly and leave at room temperature until the mixture is needed in step B.1 below.

Table 5. Diluted TURBO™ DNase Preparation

Per reaction	96 rxns (+10%)	Component
49 µL	5.4 mL	MagMAX™ TURBO™ DNase Buffer
1 µL	110 µL	TURBO™ DNase

d. Carefully aspirate and discard the supernatant without disturbing the beads, and remove the Processing Plate from the magnetic stand.

Be sure to remove all Wash Solution 2 before continuing to the TURBO DNase treatment.

e. Proceed immediately to the TURBO DNase treatment and final RNA clean-up.

B. Turbo DNase Treatment and Final RNA Clean-Up

1. Add 50 µL of Diluted TURBO DNase and shake for 10–15 min at room temp

When the Diluted TURBO DNase is added to the sample, nucleic acids are released from the RNA Binding Beads, and genomic DNA is degraded.

a. Add 50 µL Diluted TURBO DNase to each sample.

b. Shake the plate on an orbital shaker for 10–15 min at room temp at the maximum speed for larger volumes identified in step II.A.2.

2. Add 100 µL RNA Rebinding Solution and shake for 3 min to rebind the RNA

Add 100 µL of RNA Rebinding Solution (isopropanol added) to each sample and shake for 3 min at the maximum speed for larger volumes.

In this step, the RNA is bound to the RNA Binding Beads again.

3. Magnetically capture the RNA Binding Beads and discard the supernatant

a. Capture the RNA Binding Beads on a magnetic stand.

b. Carefully aspirate and discard the supernatant without disturbing the beads, and remove the Processing Plate from the magnetic stand.

4. Wash twice with 150 µL Wash Solution 2 each time

a. Add 150 µL Wash Solution 2 to each sample and shake for 1 min on an orbital shaker at the maximum speed for larger volumes.

b. Capture the RNA Binding Beads on a magnetic stand.

c. Carefully aspirate and discard the supernatant without disturbing the beads, and remove the Processing Plate from the magnetic stand.

d. Repeat steps a–c to wash with a second 150 µL of Wash Solution 2.

5. Dry the beads by shaking for 2 min

Move the Processing Plate to the shaker and shake vigorously for 2 min at the maximum speed for lower volumes identified in step II.A.2.

This dries the beads, removing residual ethanol which otherwise could interfere with downstream applications.

6. Elute the RNA in 50 µL of Elution Buffer

a. Add 50 µL Elution Buffer to each sample and shake vigorously for 3 min at the maximum speed for lower volumes.

NOTE

The elution volume is somewhat flexible; RNA can be eluted as little as 20 µL, or in >50 µL to achieve the desired final RNA concentration. The volume of Elution Buffer supplied with the kit is enough for 96 samples at 100 µL each.

b. Capture the RNA Binding Beads on a magnetic stand. The purified RNA will be in the supernatant.

c. Transfer the supernatant, which contains the RNA, to a nuclease-free container appropriate for your application.

C. Assessing RNA Yield and Purity

RNA yield

Spectrophotometry

The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm (A_{260}). We recommend using the NanoDrop® 1000A Spectrophotometer (www.nanoambion.com) because it is extremely quick and easy to use; just measure 1–2 µL of the RNA sample directly.

Alternatively, the RNA concentration can be determined by diluting an aliquot of the preparation in TE (10 mM Tris-HCl, pH 8; 1 mM EDTA) and reading the absorbance in a traditional spectrophotometer at 260 nm. 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).

$$A_{260} \times \text{dilution factor} \times 40 = \mu g\ RNA/mL$$

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

The Agilent® 2100 bioanalyzer with Caliper's RNA LabChip® Kits provides better quantitative data than conventional gel analysis for characterizing RNA. When used with Ambion RNA 6000 Ladder (Cat #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 new 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. The total RNA isolated with this kit should have an A_{260}/A_{280} ratio of 1.8–2.1. However, RNA with absorbance ratios outside of this range may still function well for qRT-PCR or other amplification-based downstream applications.

IV. Troubleshooting

A. DNA Contamination

Too much sample input

If sample input was too high (>2 x 10^6 cells, >5 mg animal tissue, or >5–10 mg plant tissue), the DNA digestion step may not be effective. To avoid DNA contamination, either reduce sample size, or include an additional DNase treatment step after RNA isolation (e.g., using the Ambion TURBO™ DNA-*free*™ Kit, Cat #AM1907).

Ambient temperature is too cool

Since the DNA digestion is done at room temperature, it is important that the ambient temperature in your lab be 20–25°C. If your lab is colder than 20°C, incubate the digestion in a 25°C incubator.

B. Well-to-Well Variation in RNA Yield

The RNA yield should be fairly uniform between wells of a 96-well plate containing the same number of cells. If a large variation in RNA yield between wells is observed, consider the following:

RNA Binding Beads were not fully resuspended/dispersed

In general, the RNA Binding Beads will disperse more easily when the temperature of the mixture is warmer than 20°C. We have also found that using the Ambion 96-well Magnetic-Ring Stand (Cat #AM10050) results in RNA Binding Bead pellets that are significantly easier to resuspend than pellets captured using other magnetic stands.

a. Make sure the RNA Binding Beads are fully resuspended before pipetting them into the Processing Plate at the start of the procedure (step III.A.1 on page 13).

b. For efficient elution of RNA from the RNA Binding Beads at the end of the procedure, make sure the beads are fully dispersed in Elution Buffer (step III.B.6 on page 15).

   If the RNA Binding Beads aggregate or fail to disperse during the final RNA elution step, it may improve RNA yield to place the Processing Plate in a 70°C incubator for 5 min and to repeat the 3 min shaking incubation before capturing the beads.

   In subsequent experiments using sample types with bead clumping problems, you can preheat the Elution Buffer to 70–80°C before adding it to the samples in step III.B.6 on page 15 to facilitate dispersion of the beads.

c. Do not overdry the beads before eluting. If the beads were inadvertently overdried, extend the shaking time in step III.B.6 on page 15 to 10 min to rehydrate the beads.

RNA Binding Beads were unintentionally lost

Since the basis of this procedure is to immobilize RNA on RNA Binding Beads, any loss of beads during the procedure will result in loss of RNA. Avoid aspirating RNA Binding Beads when removing supernatant from the captured beads. To determine whether RNA Binding Beads have been inadvertently aspirated with supernatant, it may be helpful to collect all supernatants in a single reservoir. Observe the color of the collected supernatant, if RNA Binding Beads are in the supernatant, they will tint the solution light brown.

To prevent aspiration of RNA Binding Beads in subsequent experiments, observe the following precautions:

- Use sufficient magnetic capture time.
- Aspirate supernatant slowly.
- Keep pipet tip openings away from the captured RNA Binding Beads when aspirating supernatant.

V. Appendix

A. KingFisher MagMAX-96 Total RNA Isolation Protocol Overview

The MagMAX-96 Total RNA Isolation protocol can be adapted for use with Thermo Electron's KingFisher (for 1–24 samples per run) and KingFisher 96 (for 1–96 samples per run) Magnetic Particle Processors. Downloadable protocols for these machines are available on Ambion's automation resource page on the web:

www.ambion.com/techlib/automation

The KingFisher processors completely automate the nucleic acid isolation process; here is a quick overview of how it works:

1. Pipet MagMAX-96 Total RNA Isolation Kit reagents into a KingFisher 200 µL plate(s) and insert the plate(s) into the KingFisher or KingFisher 96 instrument.

Row /Plate		Volume	Reagent(s)
A	For Cultured Cells	140 µL	Lysis/Binding Solution (isopropanol added)
		≤30 µL	Sample
		20 µL	***Bead Mix***
		190 µL	total volume
	For Animal Tissues	100 µL	Tissue homogenate in Lysis Binding Solution
		60 µL	Isopropanol
		20 µL	***Bead Mix***
		180 µL	total volume
	For Plant Tissues	50 µL	Tissue homogenate in Lysis/Binding Solution
		35 µL	Isopropanol
		20 µL	***Bead Mix***
		110 µL	total volume
B		150 µL	Wash Solution 1
C		150 µL	Wash Solution 2
D		50 µL	Diluted TURBO DNase
E		150 µL	Wash Solution 2
F		150 µL	Wash Solution 2
G		50 µL	Elution Buffer

2. Choose the MagMAX Total program using the arrow keys and start the program by pressing the START button. The approximately 25 min process is described below:

3. Total RNA is bound to RNA Binding Beads in row A (plate A the KingFisher 96) containing sample, Lysis/Binding Solution, and Bead Resuspension Mix.
4. The RNA Binding Beads are collected and released into the first Wash 1 Solution in row B (plate B).
5. The RNA Binding Beads are collected and released into the first Wash 2 Solution in row C (plate C).
6. The RNA Binding Beads are collected and released into the diluted TURBO DNase in row D (plate D).
7. The machine pauses and 100 µL of re-binding solution is added to row D (plate D) by the user to bind the RNA. Press the START button to continue.
8. The RNA Binding Beads are collected and released into the second Wash 2 Solution in row E (plate E).
9. The RNA Binding Beads are collected and lifted outside the wells of row F (plate F) to dry for 1 min.
10. The RNA Binding Beads are released into Elution Buffer in row G (plate G).
11. The used RNA Binding Beads are collected and returned to row B (plate B leaving RNA in elution Buffer in row G [plate G]).

B. 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.

C. Handling Frozen Tissue for RNA Isolation

Keep samples at –80°C until you are ready to begin

It is very important to maintain frozen tissue either at –80°C for long term storage, or on dry ice for short periods of time to allow for tissue handling and partitioning. RNA in tissue that has undergone a freeze/thaw cycle without protection by lysis reagents or RNA*later* will be degraded.

Recommendations for keeping samples frozen during partitioning

- Prepare a ***closed container*** (such as an ice chest) containing enough dry ice to create a cold vapor environment.
- To further safeguard against defrosting of small tissue fragments, prechill the container (such as a small tip box or a mortar and pestle decontaminated with RNase*Zap*) that will be used to hold the frozen tissue over dry ice during partitioning.

- Plan to use either a sharp scalpel with a handle and a nonflexible metal blade or a mortar and pestle with liquid nitrogen to fragment frozen tissue into 2.5–5 mg pieces. You will also need a pair of forceps manufactured with small 'grip' teeth near the point to grasp frozen tissue firmly. Decontaminate containers and tools with RNase*Zap*, and pre-chill in dry ice.

Instructions for partitioning tissue samples into 2.5–5 mg pieces

There are two suggested strategies for breaking frozen tissue samples into 2.5–5 mg pieces: cutting the tissue with a scalpel or freezing it in liquid nitrogen and breaking it by gently crushing in a mortar and pestle. We present detailed instructions for both of these methods below. The most important aspect of either procedure, however, is to work quickly and to keep frozen samples frozen ***completely***. Note that a 5 mg tissue fragment is quite small—roughly the size of a 2 mm cube. Figure 3 below can be used to help estimate the weight of tissue fragments.

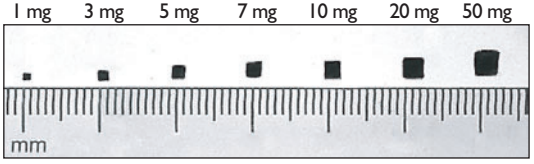


Figure 3. Estimating the Mass of Small Tissue Samples

Approximately 1–2 mm thick pieces of mouse liver tissue with the indicated weights were placed on a ruler, and photographed. Most other soft tissues are of similar density.

Use a scalpel to chop tissue into ≤2.5–5 mg pieces

Working quickly in a prechilled container that is resting on dry ice in an ice chest, slice or carve the frozen tissue into pieces (see Figure 3 as a guide). Partitioning frozen tissue into milligram pieces can be a challenge; we recommend the following technique to carve tissue effectively and rapidly. Grasp the frozen tissue firmly with chilled forceps. Position the scalpel tip on the surface of the pre-chilled container at a ~45 degree angle just in front of the tissue. Working at the edge of the tissue mass, press down firmly in a chopping motion without raising the tip of the scalpel, and chisel off a small fragment of tissue. Continue this chisel-like action until you have more tissue fragments than you need. Keep all the tissue fragments in the container resting on dry ice until you are ready to homogenize the tissue. (Keep the container closed when it is not in use.)

Break tissue by freezing in liquid nitrogen and gently crushing

Set up an ice chest with dry ice and a mortar and pestle that has been treated with RNase*Zap*. Fill the mortar with liquid nitrogen to a depth of 0.5–1 cm and drop in the tissue sample (tissue that was stored in foil

can remain in the foil or can be removed from the foil). Allow the tissue to equilibrate to the temperature of the liquid nitrogen for several seconds, and add more liquid nitrogen if necessary so that the tissue is about half immersed. Next, gently press on the tissue with the pestle to partially crush it, breaking it into 2.5–5 mg pieces in the presence of liquid nitrogen. Keep the tissue fragments frozen in the container resting on dry ice until you are ready to homogenize the tissue.

Estimate the weight of tissue fragments

Weighing tissue is the most accurate way to quantify the amount of starting material, however, it is simply not practical for most experiments. Instead, use Figure 3 as a guideline to estimate appropriately-sized tissue fragments.

D. MagMAX-96 Total RNA Isolation Kit Specifications

Kit contents

Amount	Component	Storage
1	Processing Plate with Lid	room temp
11 mL	Lysis/Binding Solution Concentrate (See section II.B starting on page 9 for instructions)	room temp
18 mL	Wash Solution 1 Concentrate (Add 6 mL 100% isopropanol before use)	room temp
55 mL	Wash Solution 2 Concentrate (Add 44 mL 100% ethanol before use)	room temp
12 mL	RNA Rebinding Concentrate (Add 6 mL 100% isopropanol before use)	room temp
10 mL	Elution Buffer	4°C or room temp
6 mL	MagMAX™ TURBO™ DNase Buffer	4°C or room temp
1.1 mL	RNA Binding Beads	4°C*
1.1 mL	Lysis/Binding Enhancer	–20°C
110 µL	TURBO DNase	–20°C

* Do not freeze the RNA Binding Beads.

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address:
  www.ambion.com/techlib/msds
- Alternatively, e-mail your request to MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.

- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

E. Quality Control

Functional testing

All kit components are tested functionally by isolating RNA from cultured cells using the procedure described in this manual. The RNA is checked for integrity and minimum yield requirements. RNA quality is assessed by Agilent bioanalyzer (28S/18S ratio = 1.5–2.0) and quantitative RT-PCR; genomic DNA contamination is assessed by quantitative RT-PCR.

Nuclease testing

Each component is tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with ^{32}P-labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated for 14–16 hr with 300 ng supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

Exonuclease activity
Meets or exceeds specification when a sample is incubated for 14–16 hr with 40 ng ^{32}P-labeled *Sau*3A fragments of pUC19 and analyzed by PAGE.

Protease testing

Meets or exceeds specification when a sample is incubated for 14–16 hr with 1 µg protease substrate and analyzed by fluorescence.