MagMAX™-96 for Microarrays Kit

High Throughput RNA Isolation for High Performance Microarray Analysis

Part Number AM1839

MagMAX™-96 for Microarrays Kit

(Part Number AM1839)

Protocol

P/N 1839M Revision F **Revision Date: November 22, 2010**

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I. Introduction

A. Product Description and Procedure Overview

The MagMAX™-96 for Microarrays Total RNA Isolation Kit (patent pending) is designed for rapid, high throughput isolation of total RNA from mammalian cells and tissues in 96-well plates. The procedure employs the robust and reliable lysis/denaturant, TRI Reagent®*, and the Ambion® MagMAX magnetic bead-based RNA purification technology. 96 samples can be processed at once with the MagMAX-96 for Microarrays Kit, however, it can also be used to efficiently isolate RNA from fewer than 96 samples. The MagMAX-96 for Microarrays Kit is optimized for use with either manual multichannel pipettors or with robotic liquid handlers.

TRI Reagent and MagMAX-96 Technology

TRI Reagent lyses cells and facilitates sample homogenization while denaturing nucleases to maintain the integrity of RNA. In the MagMAX-96 for Microarrays Kit, TRI Reagent is used in combination with our MagMAX technology. MagMAX magnetic beads bind RNA more efficiently and reproducibly than glass fiber filters, and they are quick and simple to use. By combining TRI Reagent and MagMAX technology, you get streamlined RNA purification, even in high throughput format, without sacrificing RNA quantity or quality.

One kit: two alternative procedures

The procedure is fast (<1 hr), simple, and well-suited for automation; see Figure 1 on page 2. First, mammalian cultured cells or tissues samples are homogenized in TRI Reagent, a monophasic solution containing phenol and guanidine thiocyanate. This rapidly lyses cells and inactivates nucleases. Once the tissue is homogenized in TRI Reagent, either of two alternative procedures can be followed for RNA isolation: the Spin Procedure and the No-Spin Procedure (see Table 1 on page 2).

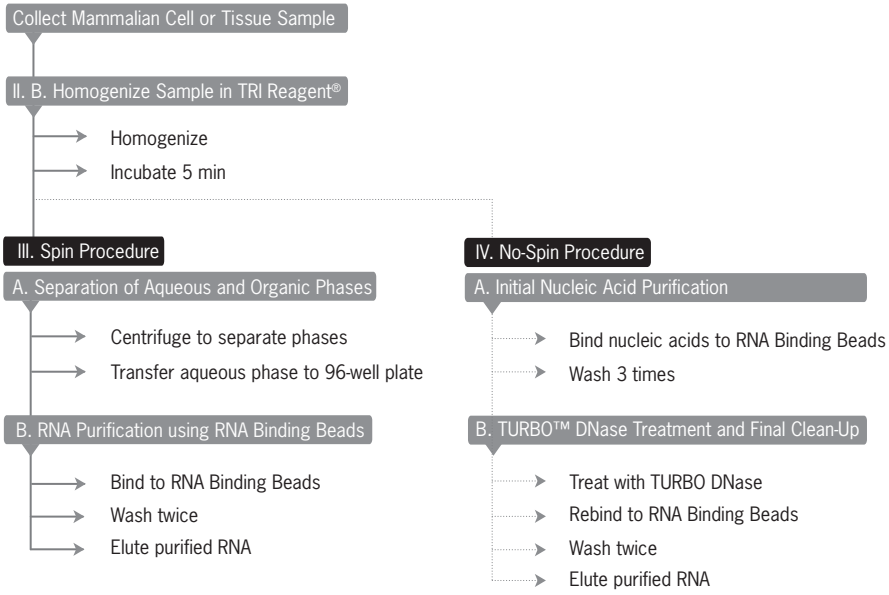
The ***Spin Procedure*** begins with the addition of bromochloropropane (BCP) and centrifugation to separate the aqueous and organic phases. The aqueous phase, containing partially purified RNA is then transferred to the wells of a 96-well plate. The RNA is then further purified using a simple magnetic bead binding and washing procedure; no DNase treatment is required. Purified RNA is eluted in 50–100 µL of low salt buffer.

Alternatively, samples can be processed using the ***No-Spin Procedure*** which starts with an initial nucleic acid purification in which magnetic beads are added directly to the homogenized sample to bind nucleic acids. Using magnetic capture, the beads and bound nucleic acids are

* TRI Reagent is a registered trademark of Molecular Research Center, Inc.

then subjected to three rapid washing steps to remove proteins and salt. In the next phase of the procedure, samples are treated with TURBO™ DNase to remove genomic DNA, and the total RNA is rebound to the magnetic beads for two final washing steps. The purified RNA is eluted in 50 µL of low salt buffer.

Figure 1. MagMAX™-96 for Microarrays Procedure Overview



High yield and high quality RNA from both procedures

Both the Spin and the No-Spin MagMAX-96 for Microarrays procedures provide high yields of pure, intact RNA that can be used directly for quantitative reverse transcriptase PCR (qRT-PCR) and microarray analysis. Figure 2 shows RNA yield and quality data that illustrates the performance of the MagMAX-96 for Microarrays RNA Isolation Kit. In this experiment, highly intact RNA purified from frozen mouse liver, consistently yielded 28S/18S ribosomal RNA (rRNA) ratios of 1.1–1.4.

Table 1. Advantages of the Spin and No-Spin Procedures

Spin Procedure	No-Spin Procedure
Better for difficult samples such as spleen and pancreas, and for RNA*later*®-treated samples	Appropriate for low fat tissues and low cellular content samples
Fewer steps: faster if only a few samples are processed	No phase separation
Compatible with tissue storage in RNA*later*	Easy to automate
No DNase treatment needed	
More tissue can be processed per sample	

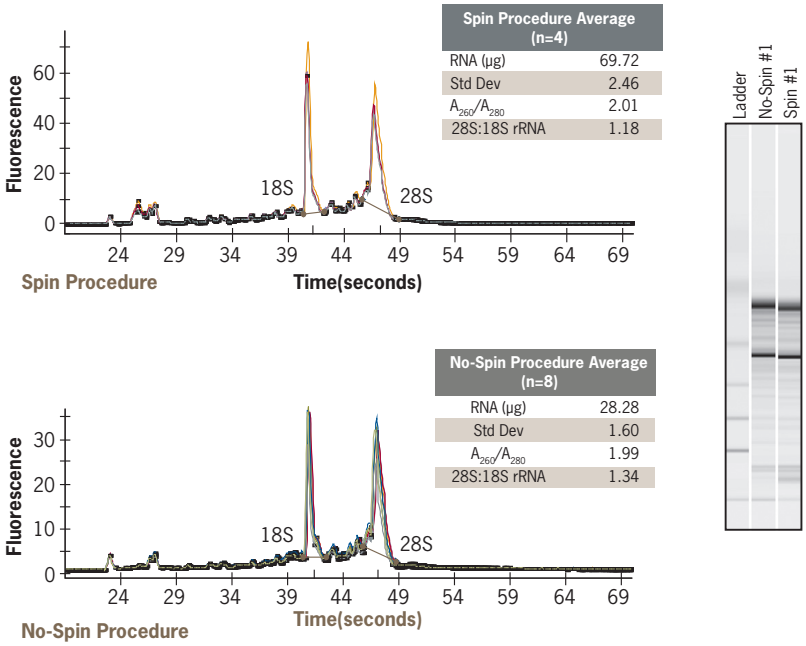


Figure 2. Consistent Yield, Purity, and Integrity of RNA Isolated with the MagMAX™-96 for Microarrays Kit.

RNA was isolated from frozen mouse liver with the MagMAX-96 for Microarrays Kit. Tissue homogenates were processed in quadruplets or octuplets using the Spin procedure (homogenate derived from 10 mg tissue per sample) or the No-Spin procedure (homogenate derived from 5 mg tissue per sample), respectively. Purified RNA (2 µL) was quantified using a NanoDrop® Spectrophotometer. The ratio of 28S to 18S rRNA was obtained by analyzing purified RNA (1 µL) using an RNA LabChip® Kit and the Agilent® 2100 bioanalyzer.

B. Kit Components and Storage Conditions

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

Amount	Component	Storage
1 ea	Processing Plate & Lid	room temp
12 mL	Lysis/Binding Solution Concentrate Add 6 mL 100% isopropanol before use.	room temp
18 mL	Wash Solution 1 Concentrate Add 6 mL 100% isopropanol before use.	room temp
72.5 mL	Wash Solution 2 Concentrate Add 58 mL 100% ethanol before use.	room temp
14 mL	Elution Buffer	room temp
6 mL	MagMAX™ TURBO™ DNase Buffer	4°C or room temp
100 mL	TRI Reagent® (four 25 mL bottles)	4°C
1.1 mL	RNA Binding Beads	4°C*
1.1 mL	Lysis/Binding Enhancer	–20°C
215 µL	TURBO™ DNase (10 U/µL)	–20°C

* ***Do not freeze the RNA Binding Beads.***

C. Required Materials Not Provided With the Kit

Lab equipment and supplies

- General laboratory equipment including vortex mixer, microcentrifuge, pipettors, and RNase-free tips
- Magnetic stand for 96-well plates: We recommend either of the Ambion® 96-well magnetic stands (P/N AM10050, AM10027) for their high strength magnets and quality design (see section I.D on page 5).
- (Optional but recommended) Multichannel pipettor
- Orbital shaker for 96-well plates such as the Barnstead/Lab-Line Titer Plate Shaker (VWR #57019-600 or Fisher #14-271-9)
- If you process fewer than 96 samples at a time, you will need additional polypropylene U bottom 96-well plates and lids.

Reagents

- 100% ethanol, ACS grade or higher quality
- 100% isopropanol, ACS grade or higher quality
- 1-bromo-3-chloropropane (BCP), e.g., MRC Cat #BP 151

D. Related Products Available from Applied Biosystems

RNase*Zap*® P/N AM9780, AM9782, AM9784	RNase Decontamination Solution. RNase*Zap* is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*.
RNA*later*® P/N AM7020, AM7021	RNA*later* is an aqueous sample collection solution that stabilizes and protects cellular RNA in intact, unfrozen tissue and cell samples. RNA*later* eliminates the need to immediately process samples or to freeze samples in liquid nitrogen. Samples can be submerged in RNA*later* for storage at RT, 4°C, or –20°C without jeopardizing the quality or quantity of RNA that can be obtained.
RNase-free Tubes & Tips See web or print catalog for P/Ns	RNase-free tubes and tips are available in most commonly used sizes and styles. They are guaranteed RNase- and DNase-free. See our latest catalog or our website (www.ambion.com) for specific information.
96-well Magnetic-Ring Stand P/N 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 multichannel 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 P/N 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 (P/N AM10050).
MessageAmp™ II aRNA Amplification Kits See web or print catalog for P/Ns	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 P/N 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. Experimental Setup and Sample Homogenization

A. Reagent and Equipment 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., RNase*Zap* Solution).

Gloves and RNase-free technique

Wear laboratory gloves; 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 180 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 100 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.C.5–6 on page 11, and steps IV.C.5–6 on page 15).

3. Before using the kit, complete the *Lysis/Binding Solution*, and *Wash Solutions 1 and 2*

a. Add 6 mL 100% isopropanol to the bottle labeled Lysis/Binding Solution Concentrate and mix well.
The mixture is called ***Lysis/Binding Solution*** in these instructions.

b. 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.

c. Add 58 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.

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

B. Sample Homogenization

1. Homogenize samples in TRI Reagent

Separate instructions are provided for sample homogenization in steps 1a (tissue) and 1b (cultured cells) below.

The maximum amount of sample homogenate that can be used in the MagMAX-96 for Microarrays procedure depends on which method is followed for the RNA isolation.

- For the ***Spin*** procedure, a maximum of 100 µL of aqueous phase after centrifugation can be used per RNA isolation reaction; we recommend preparing ~500 µL–1 mL of sample homogenate so that you can recover ≥100 µL of aqueous phase.
- For the ***No-Spin*** procedure, a maximum of 100 µL of sample homogenate can be used per RNA isolation reaction.

For samples that are not limited in supply, it is often easier to prepare more homogenate than can be used in a single RNA isolation reaction; the volume of TRI Reagent supplied with the kit is sufficient for 96 samples using 1 mL per sample.

1a. Homogenize tissue in 10–40 volumes of TRI Reagent

a. Tissue handling instructions

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

- Homogenize in TRI Reagent immediately (see *b. Tissue homogenization instructions* below).
- Freeze rapidly in liquid nitrogen (tissue pieces must be small enough to freeze in a few seconds).

NOTE

For a detailed discussion of cell and tissue disruption for RNA isolation, se Ambion Technical Bulletin #183 available at:
www.ambion.com/techlib/tb/tb_183.html

- Submerge in a tissue storage buffer such as Ambion® RNA*later* Solution (Spin procedure ***only***; samples stored in RNA*later* cannot be used in the No-Spin procedure).

Handling frozen tissue: Weigh frozen tissue, and if necessary, break it into pieces smaller than ~50 mg (keeping tissue completely frozen) and homogenize directly in TRI Reagent. Larger pieces of tissue, very hard or fibrous tissues, and tissues with a high RNase content, must typically be ground to a powder in liquid nitrogen for maximum RNA yield.

b. Tissue homogenization instructions

Homogenize samples in TRI Reagent using standard homogenization procedures. For most tissues, rotor-stator homogenizers work very well.

Spin procedure: Homogenize samples in 10–20 volumes TRI Reagent (e.g., 1 mL TRI Reagent per 50–100 mg tissue).

No-Spin procedure: For most sample types 20 volumes of TRI Reagent is appropriate (e.g., 1 mL TRI Reagent per 50 mg tissue), but for tissues that are very high in nucleases, such as spleen and pancreas, homogenize in 40 volumes of TRI Reagent (e.g., 1 mL TRI Reagent per 25 mg tissue).

1b. Homogenize cultured cells in 1 mL TRI Reagent per 10 cm^2 culture dish area, or per 5 x 10^6 cells

Do not wash cells before lysing with TRI Reagent as this may contribute to mRNA degradation.

Cells grown in monolayer: Pour off media, add 1 mL of TRI Reagent per 10 cm^2 of culture dish area, and pass the mixture through a pipette several times to lyse cells and homogenize the sample (lyse directly in the culture dish). Use the area of the culture dish, rather than the cell number, to determine the volume of TRI Reagent for lysis.

Cells grown in suspension: Pellet cells, then lyse in 1 mL of TRI Reagent per 5 x 10^6 animal, plant, or yeast cells by repeated pipetting or vortexing.

2. Incubate the homogenate for 5 min at room temp

Incubate homogenates from both tissue samples and cell cultures for 5 min at room temp. This incubation allows nucleoprotein complexes to completely dissociate.

STOPPING POINT

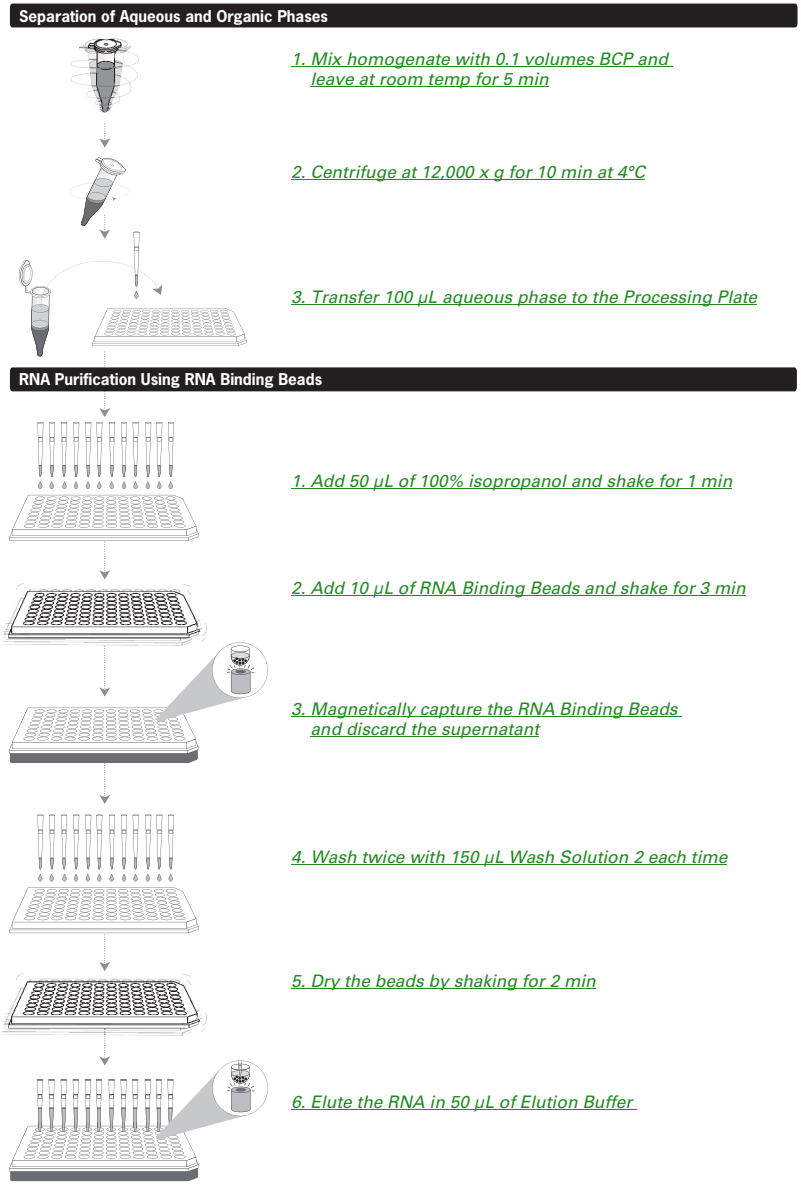
Homogenized samples can be stored at –70°C for at least one month.

Continue to either section *IV. No-Spin Procedure* starting on page 12, or to section *III. Spin Procedure* starting on page 9.

III. Spin Procedure

A. Procedure Overview

Figure 3. MagMAX™-96 for Microarrays Spin Procedure



B. Separation of Aqueous and Organic Phases

1. Mix homogenate with 0.1 volumes BCP and leave at room temp for 5 min

a. Transfer homogenized sample (from step II.B.2 on page 8) to a 1.5 mL microcentrifuge tube. Add 0.1 volumes of BCP (e.g., add 100 µL BCP to 1 mL of homogenate), and cap the tube securely.

b. Vortex at moderate speed for 5–10 seconds.

c. Store the mixture at room temperature for 5 min.

2. Centrifuge at 12,000 x g for 10 min at 4°C

Centrifuge at 12,000 x g for 10 min at 4°C to separate the sample mixture into three phases: phenol-BCP on the bottom (red), interphase in the center, and aqueous phase on the top (colorless). RNA is in the aqueous phase, while DNA and proteins are in the interphase and organic phase (phenol-BCP).

3. Transfer 100 µL aqueous phase to the Processing Plate

Transfer 100 µL of the aqueous phase to a well of the 96-well Processing Plate and continue the procedure.

You can discard the tube after removing the aqueous phase, or the other phases can be processed for protein and/or DNA isolation following the instructions available at: www.ambion.com/catalog/CatNum.php?9738

C. RNA Purification Using RNA Binding Beads

1. Add 50 µL of 100% isopropanol and shake for 1 min

a. Add 50 µL of 100% isopropanol to each sample.

b. Shake the Processing Plate for 1 min on an orbital shaker at the maximum speed for larger volumes identified in step II.A.2.

2. Add 10 µL of RNA Binding Beads and shake for 3 min

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

b. Add 10 µL of RNA Binding Beads to each sample.

c. Shake the plate for 3 min on an orbital shaker at the maximum speed for larger volumes.

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

a. 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. Using the Ambion 96-Well Magnetic-Ring Stand, the capture time is ~1–2 min.

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

IMPORTANT

To obtain pure RNA, it is important to completely remove the supernatant at this step.

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

a. Add 150 µL Wash Solution 2 (ethanol added in step II.II.3.c) 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 as in the previous step.

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 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.

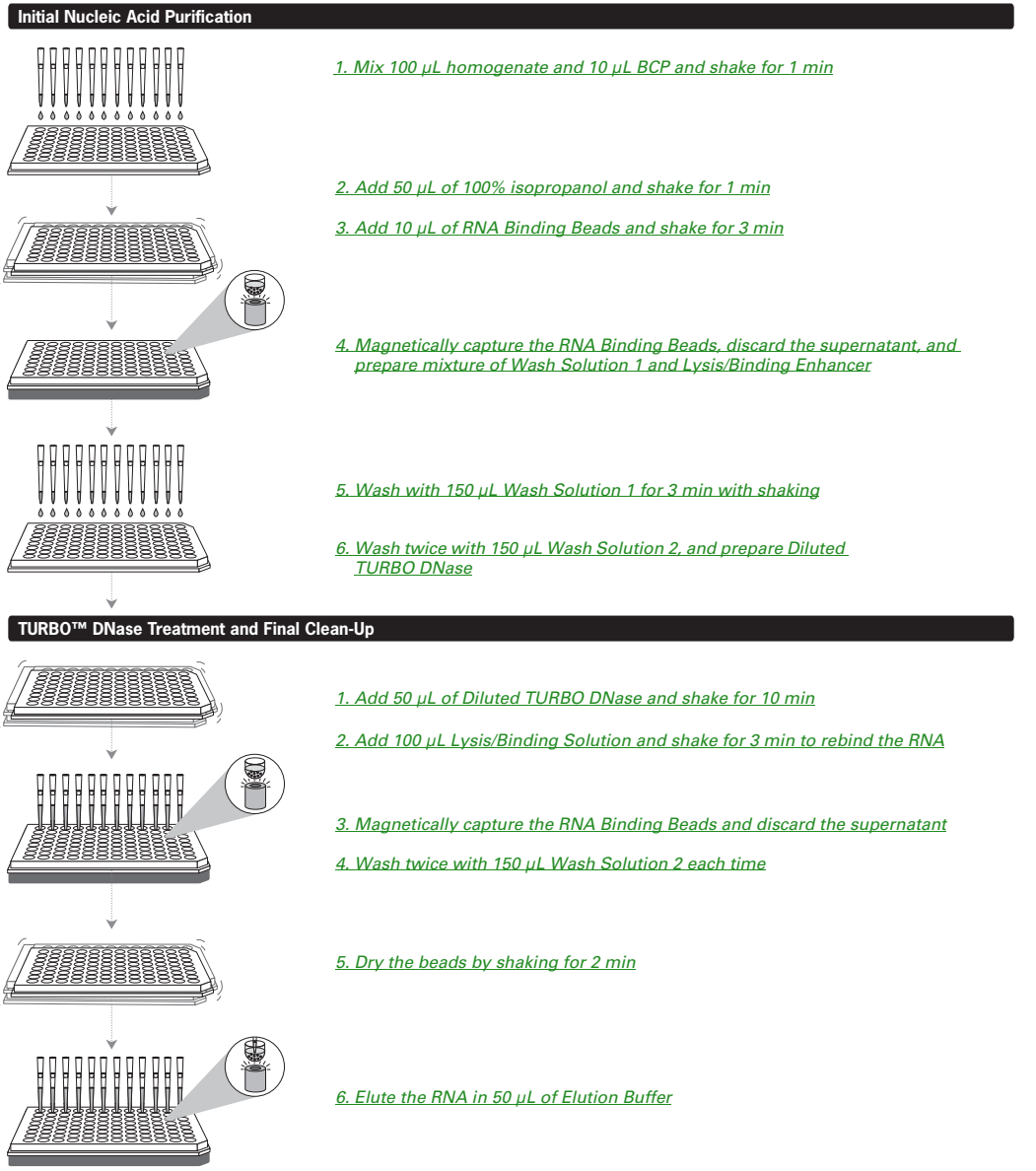
b. Capture the RNA Binding Beads on a magnetic stand. The purified RNA will be in the supernatant.

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

IV. No-Spin Procedure

A. Procedure Overview

Figure 4. MagMAX™-96 for Microarrays No-Spin Procedure



B. Initial Nucleic Acid Purification

1. Mix 100 µL homogenate and 10 µL BCP and shake for 1 min

a. For each sample to be processed, place 10 µL of BCP into a well of the 96-well Processing Plate.

b. Add 100 µL of homogenized sample from step II.B.2 on page 8 into each well containing BCP.

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

2. Add 50 µL of 100% isopropanol and shake for 1 min

a. Add 50 µL of 100% isopropanol to each sample.

b. Shake the Processing Plate for 1 min on an orbital shaker at the maximum speed for larger volumes.

3. Add 10 µL of RNA Binding Beads and shake for 3 min

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

b. Add 10 µL of RNA Binding Beads to each sample.

c. Shake the plate for 3 min on an orbital shaker at the maximum speed for larger volumes.

4. Magnetically capture the RNA Binding Beads, discard the supernatant, and prepare mixture of Wash Solution 1 and Lysis/Binding Enhancer

a. Move the Processing Plate to a 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 ~1–2 min.

b. While capturing the RNA Binding Beads, prepare 150 µL of Wash Solution 1 with Lysis/Binding Enhancer for each RNA isolation reaction according to the table below. We recommend including ~10% overage to cover pipetting error. Mix thoroughly by gently vortexing or pietting up and down a few times.

Table 2. Wash Solution 1 with Lysis/Binding Enhancer Preparation*

Component	per rxn	~100 rxns
Wash Solution 1 (isopropanol added in step II.B.3.b)	140 µL	15.4 mL
Lysis/Binding Enhancer	10 µL	1.1 mL

* We recommend preparing this solution just before use. Use it no more than 1 hr after preparing it.

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

IMPORTANT

To obtain pure RNA, it is important to completely remove the supernatant at this step.

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

a. Add 150 µL Wash Solution 1 with Lysis/Binding Enhancer to each sample and shake for 3 min on an orbital shaker at the maximum speed for larger volumes.

b. Capture the RNA Binding Beads on a magnetic stand as in step 4 on page 13.

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

IMPORTANT

As in step 4, complete removal of the supernatant is critical.

6. Wash twice with 150 µL Wash Solution 2, and prepare Diluted TURBO DNase

a. Add 150 µL Wash Solution 2 (ethanol added in step II.II.3.c) 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 step 6.e below.

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

e. 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 Diluted TURBO DNase is needed in step C.1 below.

Table 3. Diluted TURBO™ DNase Preparation

Component	per reaction	~100 reactions
MagMAX™ TURBO™ DNase Buffer	48 µL	5.3 mL
TURBO™ DNase	2 µL	220 µL

C. TURBO™ DNase Treatment and Final Clean-Up

1. Add 50 µL of Diluted TURBO DNase and shake for 10 min

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

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

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

IMPORTANT

Do not exceed 10 min for the TURBO DNase treatment.

2. Add 100 µL Lysis/Binding Solution and shake for 3 min to rebind the RNA

Add 100 µL of Lysis/Binding Solution to each sample and shake for 3 min on an orbital shaker 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 as in step B.4 on page 13.
Using the 96-Well Magnetic-Ring Stand, the capture time is ~1 min.

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 as in the previous steps.

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 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.

V. Assessing the RNA and Troubleshooting

A. Assessing RNA Yield and Purity

RNA yield

Spectrophotometry

The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm. Ambion scientists 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).

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

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 qualitative data than conventional gel analysis for characterizing RNA. When used with the Ambion RNA 6000 Ladder (P/N AM7152), this system can provide a fast and accurate size distribution profile of RNA samples. Follow the manufacturer's instructions for performing the assay.

The 28S to 18S rRNA ratio is often used as an indicator of RNA integrity. Total RNA isolated from fresh and frozen mammalian tissues using this kit usually has a 28S to 18S rRNA ratio of >1.2. Using a bioanalyzer, the RIN (RNA Integrity Number) can be calculated to further evaluate RNA integrity. A 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:

http://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.

B. Troubleshooting Poor RNA Yield or Integrity

Well-to-well variation in RNA yield

The total RNA yield should be fairly uniform between wells of a 96-well plate with the same sample type; however, RNA recovery from different samples types may vary considerably. The following troubleshooting suggestions may be helpful if large variations in RNA yield from the same sample type are observed.

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 (68°F).

a. Make sure the RNA Binding Beads are fully resuspended before pipetting them into the Processing Plate at the start of the procedure (steps IV.B.3 on page 13 and III.C.2 on page 10).

b. If RNA integrity is poor or yield is lower than expected, make sure that the RNA Binding Beads are completely resuspended during the TURBO DNase treatment at step IV.C.1 on page 15. If necessary, pipet the solution up and down to thoroughly resuspend the solution.

c. 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 (steps III.C.6 on page 11 and IV.C.6 on page 16).
   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 steps III.C.6 on page 11 and IV.C.6 on page 16 to facilitate dispersion of the beads.

d. Do not overdry the beads before eluting. If the beads were inadvertently overdried, extend the shaking time in steps III.C.6 on page 11 and IV.C.6 on page 16 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.

C. Troubleshooting DNA Contamination

With the No-Spin procedure

Try the Spin Procedure

If RNA obtained using the No-Spin procedure contains more genomic DNA contamination than your downstream assay can tolerate, you may want to try the Spin procedure with that sample type in subsequent experiments. We also recommend the Ambion TURBO™ DNA-*free*™ Kit (P/N AM1907) for removal of genomic DNA from RNA samples.

Mix the RNA Binding Beads before shaking

If RNA Binding Beads aggregate or fail to disperse in section IV.B steps 3, 5, or 6, it may improve DNA digestion to mix the RNA Binding Beads with the sample by pipetting up and down once or twice before the shaking incubation.

With the Spin procedure

Separate the aqueous and organic phases by centrifuging at 4°C

The Spin procedure includes a centrifugation at 4°C to separate homogenized samples into three distinct phases, (step III.B.2 on page 10). It is important to perform the centrifugation at 4°C for maximum separation of the DNA-containing interphase, centrifuging at room temperature typically increases DNA contamination of the aqueous phase which contains the RNA.

Avoid touching the interphase when collecting the aqueous phase

It is important to avoid touching the interphase when collecting the aqueous phase after the centrifugation (step III.B.3 on page 10).

D. Troubleshooting Impurities That Inhibit Downstream Applications

Most impurities will cause a shift in UV absorbance that can be seen by comparing a sample's UV absorbance spectrum to that of a control RNA. Any distortion in the shape of UV spectrum indicates that there are impurities in the eluted RNA. For example, protein absorbs at 280 nm, which can result in a low A_{260}/A_{280} ratio. Salt contamination may cause a peak at 230 nm.

RNA isolation reagent carryover

The Lysis/Binding and Wash Solutions contain significant amounts of proteins and salts. To avoid protein and salt carryover, remove supernatants from captured RNA Binding Beads thoroughly.

E. Troubleshooting RNA Binding Bead Carryover

If RNA Binding Beads are carried over into the eluate containing the RNA, they will cause the solution to be light brown in color. A small quantity of beads in the sample does not affect downstream applications such as RT-PCR or RNA amplification.

- See section *V.B. RNA Binding Beads were unintentionally lost* on page 18 for suggestions for avoiding bead carryover.
- To remove residual RNA Binding Beads from the purified RNA samples, place the sample plate containing the purified RNA onto the magnetic stand and recapture the RNA Binding Beads for 1 min. Then transfer the RNA solution(s) to a fresh nuclease-free plate or tubes.

VI. Appendix

A. Safety Information

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

IMPORTANT

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

To obtain Material Safety Data Sheets

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from product-specific links on our website at the following address: www.ambion.com/techlib/msds
- Alternatively, e-mail your request to: MSDS_Inquiry_CCRM@appliedbiosystems.com. Specify the catalog or part number(s) of the product(s), and we will e-mail the associated MSDSs unless you specify a preference for fax delivery.
- For customers without access to the internet or fax, our technical service department can fulfill MSDS requests placed by telephone or postal mail. (Requests for postal delivery require 1–2 weeks for processing.)

Chemical safety guidelines

To minimize the hazards of chemicals:

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

B. Quality Control

Functional testing

The MagMAX-96 for Microarrays Kit components are functionally tested by isolating RNA from mammalian tissue using the procedure described in this protocol. RNA recovery is assessed by absorbance measurements using the NanoDrop Spectrophotometer and by qRT-PCR. Integrity of the RNA is evaluated by analysis on an Agilent 2100 bioanalyzer.

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 super-coiled 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.