RNAqueous®-Micro (Cat #1931)

Instruction Manual

Manual Version 0410

Literature Citation When describing a procedure for publication using this product, we would appreciate that you refer to it as the RNAqueous®-Micro Kit.

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I. Introduction

A. Product Description

Ambion's RNAqueous®-Micro Kit is designed for isolating total RNA from micro-scale samples, including cultured cells (≤500,000 cells), tissue samples (≤10 mg), and microdissected tissue. This kit includes special reagents and instructions for RNA isolation from LCM (laser capture microdissection) samples to obtain optimal RNA recovery.

The procedure begins by lysing the sample in a lysis solution containing guanidinium thiocyanate, a strong chaotropic agent that disrupts cell membranes and rapidly inactivates ribonucleases. The lysate is then mixed with ethanol and applied to a silica-based filter that selectively binds RNA. A simple modification in the protocol allows the user to recover both large and small RNA species including tRNAs, 5S rRNA, and microRNAs.

An important feature of this system is that the silica filter, on which the RNA is purified, is very small; this allows the RNA to be thoroughly eluted in a very small volume of only ~10–20 µl. This allows for recovery of a more concentrated RNA solution than can be obtained by standard purification procedures, giving greater sensitivity in downstream assays (e.g. RT-PCR and linear amplification), without the need to further concentrate the RNA.

The kit contains reagents for optional post-elution DNase treatment to remove trace amounts of genomic DNA that could interfere with RT-PCR assays. The DNase is removed after digestion using a resin that removes DNase without heat inactivation, phenol extraction, or alcohol precipitation.

The RNAqueous-Micro RNA isolation procedure can be completed in roughly 10–15 min; the post-elution DNase treatment requires an additional 30 min.

B. Kit Components and Storage

Reagents are provided for isolating RNA from 50 samples. Store –20°C reagents in a non-frost-free freezer.

Amount	Component	Storage
50 ea	Micro Filter Cartridge Assembly (Micro Filter Cartridge in 2 ml Collection Tube)	room temp
50 ea	Micro Elution Tubes (1.5 ml)	room temp
15 ml	Wash Solution 1* Concentrate (Add 10.5 ml 100% ethanol before use)	room temp†
28 ml	Wash Solution 2/3 Concentrate (add 22.4 ml 100% ethanol before use)	4°C†
25 ml	Lysis Solution*	4°C
200 µl	LCM Additive	–20°C
55 µl	DNase (2 U/µl)	–20°C
600 µl	10X DNase I Buffer	–20°C
150 µl	DNase Inactivation Reagent	–20°C
5 ml	Elution Solution	any temp‡

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

† Store at room temp for up to 1 month. For longer term storage, store at 4°C, but warm to room temp before use.

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

Properly stored kits are guaranteed for 6 months from the date received.

C. Required Materials Not Included with the Kit

- 100% ethanol, ACS grade or better
- To isolate RNA from tissue samples, either a rotor-stator type tissue disruptor with a micro-probe, or a manual tissue disruptor is needed. The PRO-200 homogenizer, from Pro Scientific (www.proscientific.com) works well for small-scale tissue lysates (~0.5–3 ml).
- Microcentrifuge capable of at least 13,600 x g
- Heat block and/or incubator at 75°C (or 95°C for LCM samples), and 37°C (for optional DNase treatment)
- RNase-free pipette tips and pipettors
- Additional RNase-free microfuge tubes for RNA storage (if the DNase treatment is included in the procedure)

D. Related Products Available from Ambion

LCM Staining Kit Cat #1935	This kit includes reagents for staining frozen tissue sections for microdissection by a method that allows identification of target cells and preserves RNA quality during staining.
RNase*Zap*® Cat #9780–84	RNase Decontamination Solution. RNase*Zap* is simply sprayed or poured onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap*.
RNA*later*® Cat #7020, 7021	RNA*later*® is an aqueous, non-toxic 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 harvested and submerged in RNAlater 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 our web or print catalog	Ambion's 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 web site (www.ambion.com) for specific information.
RETROscript® Kit Cat #1710	First strand cDNA synthesis kit for RT-PCR. When purchased with SuperTaq™, this kit provides reagents, controls, and protocols for reverse transcription and PCR. Both oligo(dT) and random primers for cDNA priming are included, as is RNase Inhibitor.
***SuperTaq™** Cat #2050, 2052	Thermostable DNA Polymerase (includes 10X buffers and dNTPs)
***SuperTaq™ Plus** Cat #2054, (50U) Cat #2056, (250U)	Extended Range Thermostable DNA Polymerase Super Taq Plus has proof reading activity and produces significantly higher yields of PCR products than ordinary Taq polymerase (includes 10X buffers and dNTPs)
MessageAmp™ Kit Cat #1750	The MessageAmp aRNA Amplification Kit uses the powerful technique pioneered by Van Gelder and Eberwine to amplify RNA in a linear fashion. The kit employs an optimized, streamlined procedure and incorporates Ambion's MEGAscript™ technology for optimal aRNA yield.

* SuperTaq™ is made by Enzyme Technologies Limited and sold under licensing arrangements with F. Hoffmann-La Roche Ltd., Roche Molecular Systems, Inc. and The Perkin-Elmer Corporation. Ambion is a distributor of Enzyme Technologies Limited. Purchase of SuperTaq is accompanied by a limited license to use it in the Polymerase Chain Reaction (PCR) and RT-PCR process for research in conjunction with a thermal cycler whose use in the automated performance of the PCR and RT-PCR process is covered by the up-front license fee, either by payment to Perkin-Elmer, or as purchased, i.e., an authorized thermal cycler. Please Note: SuperTaq is not available for sale directly from Ambion in the United Kingdom, France, Germany, BeNeLux, Denmark, Sweden, Italy, Austria, Switzerland, Singapore, and Taiwan. Contact Enzyme Technologies LTD, Unit 4, 61 Ditton Walk, Cambridge CB5 8QD, U.K. for more information.

II. RNAqueous-Micro Protocol

A. Preparation of Materials

Add 10.5 ml ethanol to Wash Solution 1 Concentrate

Add 10.5 ml of ACS grade 100% ethanol to the bottle labeled Wash Soln 1 Concentrate. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

Add 22.4 ml 100% ethanol to Wash Solution 2/3 Concentrate

Add 22.4 ml of ACS grade 100% ethanol to the bottle labeled Wash 2/3 Concentrate. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

NOTE

Once ethanol has been added, cap the Wash Solution bottles tightly to prevent evaporation.

As you begin the procedure:

- For each sample, label a Micro Filter Cartridge Assembly and a Micro Elution Tube.
- Warm Wash Solution 2/3 to room temperature.
- Heat an aliquot of Elution Solution in a tightly closed RNase-free tube (not supplied with kit) in a heat block set to 75°C. The volume needed depends on the number of samples processed; each sample is typically eluted in 20 µl, however we recommend heating at least 50 µl of Elution Solution per sample to allow for ease of handling.

B. Sample Preparation and Disruption

1. Sample preparation

Cultured cells (≤5 x 10^5 cells):

- Count cells; pellet up to 500,000 cells by centrifugation, and thoroughly remove supernatant by aspiration.
- The cell pellet can be washed with 1X phosphate buffered saline (PBS) prior disruption, but this is not essential. To wash cells in PBS, resuspend in 1 ml PBS, repellet the cells, and thoroughly remove the fluid.
- Proceed immediately to sample disruption (next step).

Solid tissue (≤10 mg):

To obtain high quality RNA, minimize the time between tissue collection and RNase inactivation. Options for RNase inactivation are as follows:

- immediately disrupt fresh tissue in Lysis Solution.
- snap freeze the tissue in liquid nitrogen, and store at –70°C or colder.
- place samples in RNA*later*®, and store up to 1 week at 25°C, up to 1 month at 4°C, or indefinitely at –20°C.

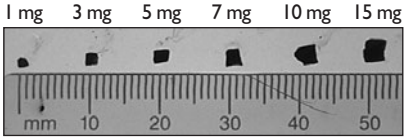

Figure 1. Estimating Mass of Small Tissue Samples

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

2. Disrupt sample in at least 100 µl Lysis Solution

Cultured cells:

Resuspend cell pellet (up to ~500,000 cells) by vortexing vigorously in at least 100 µl Lysis Solution.

Solid tissue:

Using a rotor-stator type mechanical homogenizer with a micro-probe, or a small manual tissue disruptor, thoroughly disrupt small pieces of tissue in ≥10 volumes of Lysis Solution per mass of tissue (e.g. 100 µl Lysis Solution for 10 mg of tissue). For tissue samples smaller than 10 mg, use 100 µl of Lysis Solution (this will be in excess of 10 volumes) in order to have a large enough volume for efficient disruption.

Generally, lysate from at least 5 mg of tissue can be processed per Micro Filter Cartridge. More can be processed, but the increase in yield may not be proportional to the increase in amount of tissue. The maximum amount depends on type of tissue, on how thoroughly it was disrupted, and on whether it was treated with RNA*later*, which may increase the tendency to clog the filter. The maximum amount of tissue that can be processed is limited because of filter clogging, not by the binding capacity of the filter (for further details on avoiding filter clogging, see section IV.C on page 16).

C. RNA Isolation Protocol

1. Add one-half volume of 100% ethanol to the lysate and mix

For a standard prep of 100 µl of lysate, add 50 µl of 100% ethanol, and vortex briefly but thoroughly.

If desired, centrifuge the tube briefly to collect the sample at the bottom of tube.

NOTE

The basic RNAqueous-Micro procedure does not efficiently recover small RNAs such as tRNA, 5S rRNA, and micro RNA, but can be modified for recovery these species by adding 1.25 volumes of ethanol in step 1, instead of adding 0.5 volumes of ethanol. This modification may result in more variation in RNA yields compared to the standard protocol.

2. Pass the lysate/ethanol mixture through a Micro Filter Cartridge Assembly 150 µl at a time

a. Load the lysate/ethanol mixture (up to 150 µl) onto a Micro Filter Cartridge Assembly and close the cap.

b. Centrifuge for ~10 sec at maximum speed or until all of the mixture has passed through the filter. Longer centrifugation times may be needed to filter the lysate from tissue samples.

For lysate/ethanol mixtures >150 µl, load and filter the first 150 µl, then repeat with additional aliquots until the entire sample has passed through the filter. The Collection Tube has a capacity of ~700 µl when assembled with a Micro Filter Cartridge; if more than 150 µl of lysate/ethanol mixture is filtered, empty the Collection Tube before proceeding.

The RNA is now bound to the filter in the Micro Filter Cartridge.

NOTE

All centrifugation in the following steps should be done at 13,400–16,500 x g. This is typically 12,000–13,200 rpm for standard microcentrifuges

3. Wash the filter with 180 µl Wash Solution 1

a. Open the Micro Filter Cartridge, add 180 µl of Wash Solution 1 (working solution mixed with ethanol) to the filter and close the cap.

b. Centrifuge for ~10 sec to pass the solution through the filter.

4. Wash the filter with 2 x 180 µl Wash Solution 2/3

a. Open the Micro Filter Cartridge, add 180 µl of Wash Solution 2/3 (working solution mixed with ethanol) to the filter and close the cap.

b. Centrifuge for ~10 sec to pass the solution through the filter.

c. Repeat with a second 180 µl aliquot of Wash Solution 2/3.

5. Discard the flow-through and centrifuge the filter for 1 min at max speed

a. Open the Micro Filter Cartridge assembly, remove the filter cartridge from the Collection Tube, and pour out the flow-through.

b. Replace the Micro Filter Cartridge into the same Collection Tube, close the cap, and centrifuge at maximum speed for 1 min to remove residual fluid and dry the filter.

6. Elute the RNA into a Micro Elution Tube with 2 x 5–10 µl preheated Elution Solution

a. Label a Micro Elution Tube (1.5 ml tubes provided with the kit) and transfer the Micro Filter Cartridge into it.

b. Apply 5–10 µl of Elution Solution, preheated to 75°C, to the center of the filter. Close the cap and store the assembly for 1 min at room temp. Centrifuge the assembly for ~30 sec to elute the RNA from the filter.

Tension from the hinge of the Micro Elution Tube can occasionally cause the cap to pop off during the elution spin. To minimize the chance of this happening, bend the cap hinge back and forth several times, then press the cap securely onto the Micro Filter Cartridge.

c. Repeat with a second 5–10 µl aliquot of preheated Elution Solution, collecting the eluate in the same Micro Elution Tube.

NOTE

The exact volume of Elution Solution used is not critical and may be increased if desired. In general, ~75–85% of the RNA will be recovered from samples derived from up to ~100,000 cells (or 3 mg tissue) using 2 x 5 µl of Elution Solution, and ≥85% will be recovered using 2 x 10 µl of Elution Solution. A larger volume of Elution Solution may be required for thorough elution of the RNA from larger samples, especially tissue samples >3 mg.

D. (optional) DNase I Treatment and DNase Inactivation

NOTE

This DNase treatment is optional but recommended if the RNA will be used in RT-PCR assays or other applications where it is desirable to remove trace amounts of contaminating genomic DNA.

1. Add 1/10 volume of 10X DNase I Buffer and 1 µl of DNase I

Add 1/10th volume 10X DNase I Buffer (e.g., 2 µl for RNA eluted in 20 µl) and 1 µl of DNase I (provided with the kit) to the sample and mix gently but thoroughly.

2. Incubate 20 min at 37°C

- Incubate the DNase reaction 20 min at 37°C.
  A 30 min incubation may be needed for treating RNA derived from >100,000 cells or from tissue samples larger than ~3 mg.
- Remove the DNase Inactivation Reagent from –20°C and allow it to thaw at room temp during this incubation.

3. Add 2 µl or 1/10 volume DNase Inactivation Reagent, mix well, and leave at room temp for 2 min

a. Vortex the DNase Inactivation Reagent vigorously to completely resuspend the slurry.

b. Use 2 µl or 1/10 volume DNase Inactivation Reagent, whichever is greater. For example, if the RNA volume is 50 µl, and 1 µl DNase I and 5 µl of DNase Buffer were used in step 1, add 5.6 µl of DNase Inactivation Reagent.

NOTE

The DNase Inactivation Resin may become difficult to pipette after multiple uses due to depletion of fluid from the interstitial spaces. If this happens, add a volume of Elution Solution equal to approximately 10–20% of the remaining DNase Inactivation Reagent and vortex well to recreate a pipettable slurry.

c. Store the reaction at room temp for 2 min, vortexing once during this interval to disperse the DNase Inactivation Reagent.

4. Pellet the DNase Inactivation Reagent and transfer the RNA to a fresh tube

Centrifuge the reaction 1.5 min at maximum speed to pellet the DNase Inactivation Reagent. Transfer the RNA to a fresh RNase-free tube (not supplied with the kit) and store at –20°C.

III. RNAqueous-Micro Protocol for LCM

This section provides instructions for RNA isolation from LCM samples using a procedure optimized to obtain the maximal yield and quality. For RNA isolation from samples from sources other than LCM, follow the protocol in section II starting on page 4.

A. Preparation of Materials

Add 10.5 ml ethanol to Wash Solution 1 Concentrate

Add 10.5 ml of ACS grade 100% ethanol to the bottle labeled Wash Soln 1 Concentrate. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

Add 22.4 ml 100% ethanol to Wash Solution 2/3 Concentrate

Add 22.4 ml of ACS grade 100% ethanol to the bottle labeled Wash 2/3 Concentrate. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

NOTE

Once ethanol has been added, cap the Wash Solution bottles tightly to prevent evaporation.

As you begin the procedure:

- For each sample, label a Micro Filter Cartridge Assembly and a Micro Elution Tube.
- Warm Wash Solution 2/3 to room temperature.
- Heat an aliquot of Elution Solution in a tightly closed RNase-free tube (not supplied with kit) in a heat block set to 95°C. The volume needed depends on the number of samples processed; each sample is typically eluted in 20 µl, however we recommend heating at least 50 µl of Elution Solution per sample to allow for ease of handling.

B. RNA Isolation Protocol from LCM Samples

Microdissected sample preparation

For extraction of RNA, frozen sections (rather than paraformaldehyde or formalin fixed, paraffin-embedded sections) are generally used in order to avoid crosslinking and otherwise damaging the RNA. To minimize RNA degradation during staining, avoid placing the sections in water or aqueous stain solutions; Ambion's LCM Staining Kit (Cat #1935) is recommended.

1. Drop sample into 100 µl Lysis Solution and incubate for 30 min at 42°C

Using sharp forceps, peel off the thermoplastic film containing the captured cells and drop it into a 0.5 ml microfuge tube containing 100 µl of Lysis Solution. Make sure that the sample is completely immersed in Lysis Solution.

a. Incubate the sample for 30 min at 42°C.

b. Vortex briefly to mix. Briefly centrifuge to collect the fluid at the bottom of the tube.

2. Prewet the Filter with 30 µl of Lysis Solution for ≥5 min

a. Prewet the Micro Filter Cartridge Assembly by applying 30 µl of Lysis Solution to the center of the filter and allow it to soak while performing the next two steps.

b. After completing steps 3 and 4 below, or after 5 min, centrifuge the prewetted filter for ~30 seconds at top speed to remove liquid.

3. Add 3 µl of LCM Additive to the lysate and mix

a. Add 3 µl of the LCM Additive to the lysate and mix well by briefly vortexing.

b. Briefly centrifuge to collect the fluid at the bottom of the tube.

4. Add 100% ethanol to recover either only large RNA species or all RNA

The amount of ethanol added to the lysate mixture determines what size of RNA will be captured on the filter. Instructions are provided below for recovering only RNA species larger than ~75 bases, or for recovering both large and small RNA species, including tRNAs and microRNAs.

- To recover ***only large*** RNA species:
  Add 0.5 volumes (52 µl) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing.
- To recover ***large and small*** RNA species:
  Add 1.25 volumes (129 µl) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing. (Yields of total RNA using this method may vary from those obtained using the above method.)

5. Pass the lysate mixture through a prepared Micro Filter Cartridge Assembly

Be sure to remove the Lysis Solution used to prewet the filter by centrifugation (step 2.b) before applying the lysate mixture.

a. Load the entire lysate/ethanol mixture onto the prepared Micro Filter Cartridge Assembly and close the cap.

b. Centrifuge for 1 min at 10,000 x g to bind the RNA to the filter.

6. Wash with180 µl of Wash Solution 1

a. Add 180 µl of Wash Solution 1 (working solution mixed with ethanol) to the filter and close the cap.

b. Centrifuge for 1 min at 10,000 x g to pass the solution through the filter.

NOTE

All centrifugation in the following steps should be done at 13,000 X g, or maximum speed in a microcentrifuge.

7. Wash the filter with 2 x 180 µl Wash Solution 2/3

a. Open the Micro Filter Cartridge, add 180 µl of Wash Solution 2/3 (working solution mixed with ethanol) to the filter and close the cap.

NOTE

Sometimes a precipitate will form in Wash Solution 2/3 over time. Avoid removing the crystals of precipitated material at the bottom of the bottle when removing the Wash Solution for use. There is no need to redissolve the precipitate.

b. Centrifuge for ~30 sec to pass the solution through the filter.

c. Repeat with a second 180 µl aliquot of Wash Solution 2/3.

8. Discard the flow-through and centrifuge the filter for 1 min

a. Open the Micro Filter Cartridge assembly, remove the filter cartridge from the Collection Tube, and pour out the flow-through.

b. Replace the Micro Filter Cartridge into the same Collection Tube, close the cap, and centrifuge the assembly for 1 min to remove residual fluid and dry the filter.

9. Elute the RNA in 2 x 8–10 µl of Elution Solution

a. Label a Micro Elution Tube (1.5 ml tubes provided with the kit) and transfer the Micro Filter Cartridge into it.

b. Apply 8–10 µl of Elution Solution, preheated to 95°C, to the ***center of the filter***. Since accurate pipetting of hot liquids can be difficult, pipet the Elution Solution up an down every time a new tip is used.

c. Close the cap and store the assembly for ***5 min*** at room temp.

d. Centrifuge the assembly for 1 min to elute the RNA from the filter.

Tension from the hinge of the Micro Elution Tube can occasionally cause the cap to pop off during the elution spin. To minimize the chance of this happening, bend the cap hinge back and forth several times, then press the cap securely onto the Micro Filter Cartridge.

e. Repeat steps b–d with a second 8–10 µl of preheated Elution Solution and collect the eluate in the same Micro Elution Tube.

NOTE

The exact volume of Elution Solution used is not critical and may be adjusted according to user preference. In general, 85–90% of the RNA bound to the filter will be recovered using 2 x 8–10 µl of Elution Solution.

C. (optional) DNase I Treatment and DNase Inactivation

NOTE

This DNase treatment is optional but recommended if the RNA will be used in RT-PCR assays or other applications where it is desirable to remove trace amounts of contaminating genomic DNA.

1. Add 1/10 volume of 10X DNase I Buffer and 1 µl of DNase I

Add 1/10th volume 10X DNase I Buffer (e.g. 2 µl for RNA eluted in 20 µl) and 1 µl of DNase I (provided with the kit) to the sample and mix gently but thoroughly.

2. Incubate 20 min at 37°C

- Incubate the DNase reaction for 20 min at 37°C.
  A 30 min incubation may be needed for treating RNA derived from >100,000 cells or from tissue samples larger than ~3 mg.
- Remove the DNase Inactivation Reagent from –20°C and allow it to thaw at room temp during this incubation.

3. Add 2 µl or 1/10 volume DNase Inactivation Reagent, mix well, and leave at room temp for 2 min

a. Vortex the DNase Inactivation Reagent vigorously to completely resuspend the slurry.

b. Use 2 µl or 1/10 volume DNase Inactivation Reagent, whichever is greater. For example, if the RNA volume is 50 µl, and 1 µl DNase I and 5 µl of DNase Buffer were used in step 1, add 5.6 µl of DNase Inactivation Reagent.

NOTE

The DNase Inactivation Resin may become difficult to pipette after multiple uses due to depletion of fluid from the interstitial spaces. If this happens, add a volume of Elution Solution equal to approximately 10–20% of the remaining DNase Inactivation Reagent and vortex well to recreate a pipettable slurry.

c. Store the reaction at room temp for 2 min, vortexing once during this interval to disperse the DNase Inactivation Reagent.

4. Pellet the DNase Inactivation Reagent and transfer the RNA to a fresh tube

Centrifuge the reaction 1.5 min at maximum speed to pellet the DNase Inactivation Reagent. Transfer the RNA to a fresh RNase-free tube (not supplied with the kit) and store at –20°C.

IV. Troubleshooting

A. Contaminating Genomic DNA Is Detected by RT-PCR

A minus-RT control, one in which the reverse transcriptase is omitted, should be run with every reaction. If PCR product is amplified in the minus-RT control, it is probable that the sample contains genomic DNA. The following suggestions address this result:

1. If the RNA was not treated with DNase, carry out the DNase treatment described in section *II.D. (optional) DNase I Treatment and DNase Inactivation* on page 7.
2. Repeat the DNase treatment a second time.
3. To avoid amplification of genomic DNA, even if it is present, one of two approaches is suggested for primer design.

   Span an exon-exon boundary: Choose primers that span an exon-exon boundary in the target mRNA so that one-half of the primer hybridizes to the 3' end of one exon and the other half to the 5' end of the next exon. Primers designed this way will not anneal to genomic DNA, but will anneal to cDNA synthesized from spliced mRNAs. We recommend Applied Biosystems' Gene-Expression-Assays™, many of which are designed to span exon-exon junctions.

   Flank a large intron: Choose primers on either side of a large intron so that, under the cycling conditions used, no amplification will occur from the genomic DNA template.

B. RNA Is Partially Degraded and/or RNA Yield Is Lower than Expected

If analysis of the major ribosomal RNA peaks (i.e. 18S and 28S rRNA for mammalian RNA) using a sensitive assay such as microfluidic capillary electrophoresis (e.g. Agilent bioanalyzer) indicate that the RNA yield is low or the RNA is partially degraded, consider the following suggestions.

RNA degradation in frozen tissue sections occurred during the staining process

If RNA degradation is encountered with microdissected samples, it may have occurred during the prestaining/staining/post-staining steps used to process the frozen section. To avoid RNA degradation during tissue processing, avoid placing sections in water or aqueous stain solutions at any time; exposing the sections to water allows the endogenous ribonucleases to become active. Ambion's LCM Staining Kit (Cat #1935) has been optimized for recovery of intact RNA from frozen tissue sections and is recommended.

Tissue sections were fixed in formalin or paraformaldehyde

The RNA quality will suffer if the tissue was fixed in paraformaldehyde or formalin prior to sectioning, because this fixation method crosslinks and damages nucleic acids. Use of frozen sections, instead of aldehyde-fixed sections, is recommended for recovering intact RNA from microdissected tissue sections.

RNA was incubated improperly during DNase treatment

Higher temperatures or longer incubation times during DNase treatment may result in RNA degradation.

LCM sample was incubated improperly during lysis

Samples derived from LCM require an extended incubation for thorough lysis; if samples were incubated for less than the recommended 30 min, this may result in lower RNA yields due to incomplete removal of tissue from the LCM cap.

Sample was not disrupted thoroughly and immediately in Lysis Solution

Inactivation of the endogenous RNases in the sample requires that the sample be thoroughly disrupted in the Lysis Solution. If a smooth surface was used to disrupt the tissue, disruption may be improved by using a conical ground-glass homogenizer with a rough grinding surface. If the sample disruption was done manually, for example using a plastic pestle, try using a mechanical tissue homogenizer or a bead-beater to improve the efficiency of sample disruption.

It is important to inactive RNases as quickly as possible after sample collection to avoid RNA degradation.

The LCM process should be done as quickly as possible and the samples should be lysed as soon as possible after LCM. If multiple samples are being processed, it is best to perform LCM on only a few at a time so that they can be lysed more quickly.

Problems occurred with RNA elution from the Micro Filter Cartridge

a. Be sure to elute RNA with ***heated*** Elution Solution and allow the Elution Solution to soak the filter before centrifuging the Micro Filter Cartridge Assembly to elute the RNA.

b. If the elution volume was less than 2 x 10 µl, some of the RNA may remain on the filter in the Micro Filter Cartridge. To maximize RNA recovery elute with 2 x 10 µl Elution Solution (step II.C.6 on page 7).

   Larger volumes of Elution Solution (~2 x 20 µl) may be needed to maximize recovery of RNA from tissue samples, especially if they exceeded ~3 mg.

c. Since small volumes are used for elution, it is important to pipet the Elution Solution on the center of the filter to ensure that the entire filter is soaked. If Elution Solution was pipeted on the side of the tube, the Elution Solution may not have come in complete contact with the filter, resulting in poor RNA yields.

C. Filter Clogged

Add extra Lysis Solution to lysate

Adding extra Lysis Solution to the lysate will dilute the sample and improve passage through the filter.

Increase the ethanol added to the lysate

In step II.C.1 of the protocol, add 1.25 volumes of ethanol instead of 0.5 volumes; this will recover small and large RNAs, but in most cases will resolve clogging issues.

Add 50 µl of 100% ethanol to a clogged filter to help pass solution through

To recover a sample from a clogged filter, add 50 µl of 100% ethanol to the filter and centrifuge to pass it through; this will not compromise the RNA integrity, but may reduce yield. If the filter is severely clogged, even this may not be effective.

V. Appendix

A. RNAqueous-Micro Kit Specifications

Kit contents and storage:

Reagents are provided for isolating RNA from 50 samples. Store –20°C reagents in a non-frost-free freezer.

Amount	Component	Storage
50 ea	Micro Filter Cartridge Assembly (Micro Filter Cartridge in 2 ml Collection Tube)	room temp
50 ea	Micro Elution Tubes (1.5 ml)	room temp
15 ml	Wash Solution 1* Concentrate (Add 10.5 ml 100% ethanol before use)	room temp †
28 ml	Wash Solution 2/3 Concentrate (add 22.4 ml 100% ethanol before use)	4°C†
25 ml	Lysis Solution*	4°C
200 µl	LCM Additive	–20°C
55 µl	DNase (2 U/µl)	–20°C
600 µl	10X DNase I Buffer	–20°C
150 µl	DNase Inactivation Reagent	–20°C
5 ml	Elution Solution	any temp‡

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

† Store at room temp for up to 1 month. For longer term storage, store at 4°C, but warm to room temp before use.

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

To obtain Material Safety Data Sheets:

- Material Safety Data Sheets (MSDSs) can be printed or downloaded from our website by going to the following address and clicking on the link for the RNAqueous-Micro Kit:
  www.ambion.com/techlib/msds
- Alternatively, email us at MSDS@ambion.com to request MSDSs by e-mail, fax, or ground mail.
  Specify the Ambion catalog number of the kit(s) for which you want MSDSs and whether you want to receive the information by e-mail, fax, or ground mail. Be sure to include your fax number or mailing address as appropriate. If the mode of receipt is not specified, we will e-mail the MSDSs.
- Customers without internet access can contact our technical service department by telephone, fax, or ground mail to request MSDSs (see contact information on the back of this booklet).

B. Quality Control

Functional testing

≥200 ng of total RNA is obtained from 10,000 cells using the RNAqueous-Micro Kit. The 28S:18S rRNA ratio is ≥1.8 as determined by capillary electrophoresis on an Agilent bioanalyzer. qRT-PCR analysis shows that the DNA contamination is less than 0.05% of the input RNA, and that one cell equivalent can be detected. The LCM procedure is verified by isolating ≥1 ng of RNA from 100 cells.

Nuclease testing

Each component is tested in Ambion's rigorous nuclease assays:

RNase activity

None detected after incubation with ^{32}P-labeled RNA; analyzed by PAGE.

Non-specific endonuclease/nickase activity

None detected after incubation with supercoiled plasmid DNA; analyzed on a 1% agarose gel.

Exonuclease activity

None detected after incubation with ^{32}P-labeled *Sau*3A fragments of pUC19; analyzed by PAGE.