# Mouse RiboPure™-Blood RNA Isolation Kit

(Part Number AM1951)

*Protocol*

**P/N 1951M Revision B** **Revision Date: August 29, 2008**

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# I. Introduction

## A. Product Description

The Mouse RiboPure™-Blood RNA Isolation Kit is designed to isolate high yields of high quality total RNA—optionally including small RNAs such as microRNA (miRNA)—from mouse and rat whole blood. The kit is designed for processing 0.25–0.5 mL blood samples; with minor procedural modifications lower blood volumes can be used. Microcentrifuge tubes pre-loaded with RNA*later*® are included for immediate stabilization of RNA profiles in the sample. The stabilized samples can be stored for up to 3 days at room temperature, or for prolonged periods at –20°C before RNA isolation.

The RNA isolation method is based on lysis of the blood in a guanidinium-based solution, followed by phenol/chloroform extraction, and final purification using glass fiber filter technology.

RNA can be isolated from whole blood in ~35 min using the Mouse RiboPure-Blood Kit. Typically, 30–55 µg of total RNA (including miRNA) is obtained from 0.5 mL mouse blood. (RNA yields from mouse blood are considerably higher than from human blood.) Yields of RNA from rat blood are approximately 18–40 µg from 0.5 mL blood.

RNA isolated using the Mouse RiboPure-Blood Kit is highly pure and can be used for most downstream applications, including RT-PCR, qRT-PCR, and microarray analysis.

## B. Procedure Overview

### Sample collection and RNA stabilization

Collect a 0.25–0.5 mL sample of mouse or rat whole blood, by the method of your choice, such as cardiac puncture, orbital or tail bleed, or aspiration from the chest cavity after severing the hepatic artery. Add the sample to a supplied microcentrifuge tube which is pre-loaded with RNA*later* and mix thoroughly (Figure 1 on page 2). Samples in RNA*later* are stable for up to 3 days at room temperature or 4°C, or for prolonged periods at or below –20°C.

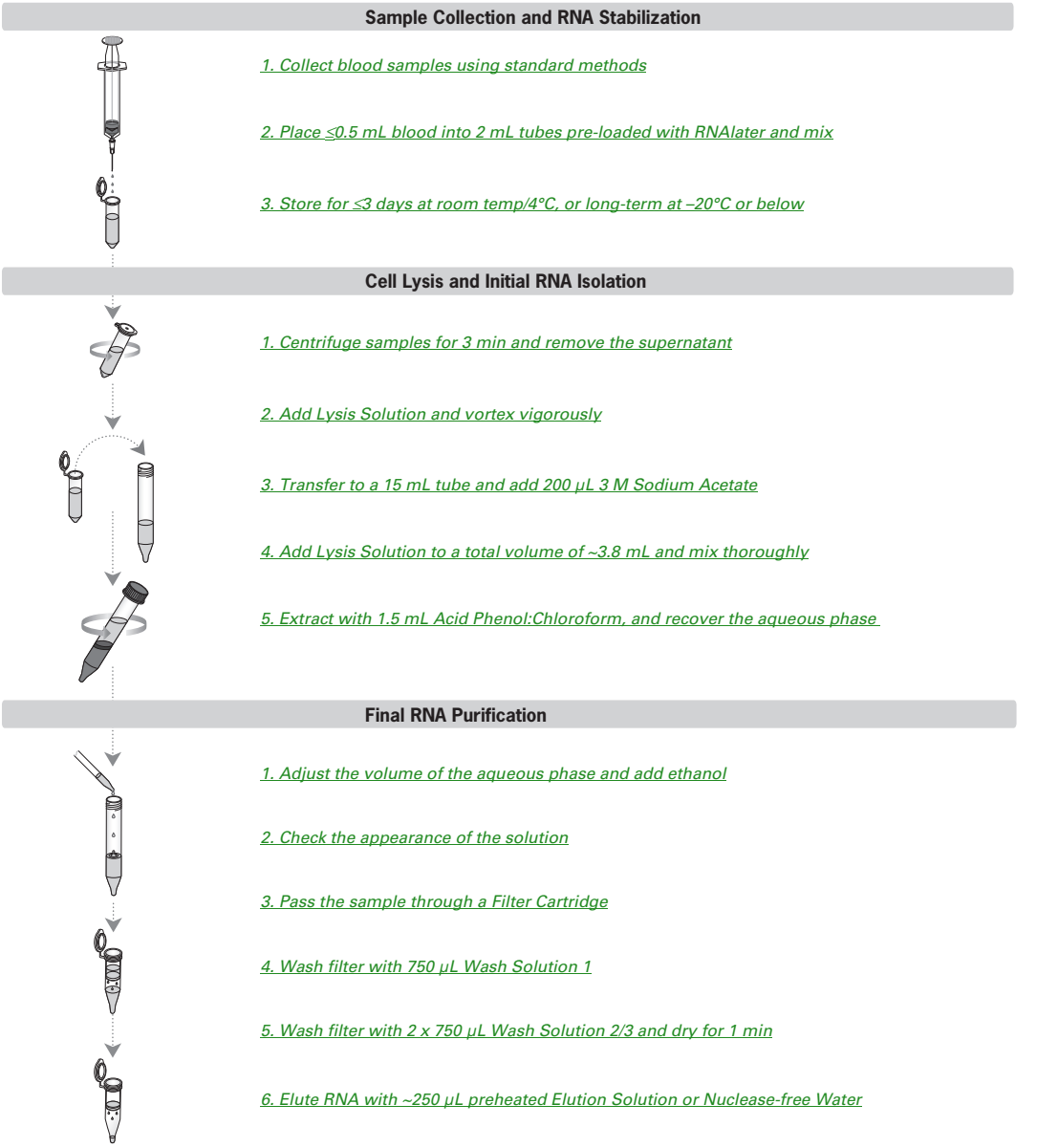
NOTE

*Do not add more than 0.5 mL of blood to the tube. Figure 3 on page 8 shows the maximum fill-line on the pre-loaded tube of RNAlater.*

### Cell lysis and initial RNA isolation

Centrifuge and remove the supernatant, then lyse the sedimented blood in Lysis Solution (guanidinium-based). Next, extract the lysate with acid phenol/chloroform to remove most of the hemoglobin and other blood proteins.

Figure 1. Mouse RiboPure™-Blood RNA Isolation Procedure Overview



### Final RNA purification

RNA in the recovered aqueous phase is further purified using a glass fiber filtering procedure. For recovery of total RNA which includes miRNA and other small RNAs, the aqueous phase is diluted with Nuclease-free Water, adjusted with ethanol, and filtered through the glass fiber Filter Cartridge provided. Users who don't require small RNA recovery can skip the dilution step and filter with a smaller overall volume. We recommend using a vacuum filtration apparatus to filter the relatively large preparation volumes, but they can alternatively be processed by multiple centrifugation passes into microcentrifuge tubes.

The RNA bound to the glass fiber filter is washed three times and then eluted with 80°C Elution Solution. Typical recovery of RNA is shown in Figure 2.

***For ≤0.25 mL blood samples***, use reduced volumes of RNA*later*, Lysis Solution, Acid Phenol/Chloroform, and Elution Solution as described throughout the procedure.

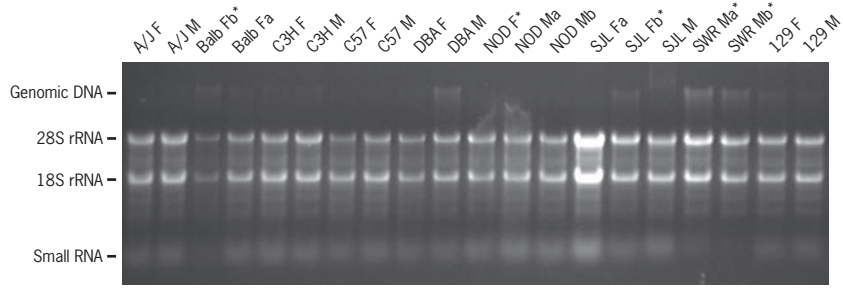



Figure 2. RNA isolated using the Mouse RiboPure™ -Blood RNA Isolation Kit

Total RNA, including miRNA and other small RNAs, was isolated from ~0.5 mL blood samples from mice of the indicated inbred strains using the Mouse RiboPure-Blood Kit. Blood was collected at The Jackson Laboratory and mixed with RNA*later*®, then shipped to Ambion at ambient temperature and stored at –20°C until processing. Samples were not treated with DNase. An aliquot (~5%) of the eluted RNA was run on a denaturing agarose gel. The SWR Mb sample was processed for total RNA depleted of small RNA, and was eluted in 125 µL instead of 250 µL. RNA samples marked with * were extracted from ~0.2–0.3 mL blood.

### Optional treatments of purified RNA

Trace levels of genomic DNA can be removed by DNase treatment; we recommend using the Ambion DNA-*free*™ Kit (P/N AM1906).

Since the RNA is obtained from whole blood, it contains high levels of globin mRNA, which can compromise sensitivity for microarray detection. Globin mRNA levels can be reduced ~95% by treating the eluted RNA with Ambion GLOBINclear™–Mouse/Rat Kit (P/N AM1981), which uses a subtractive hybridization method to deplete alpha- and beta-globin mRNAs.

## C. Kit Components and Storage Conditions

The Mouse RiboPure-Blood Kit contains reagents to isolate RNA from 25, 0.5 mL whole blood samples from mouse or rat.

| Amount | Component | Storage |
|---|---|---|
| 2 x 25 mL | Acid-Phenol:Chloroform* | 4°C |
| 6 mL | 3M Sodium Acetate pH 5.5 | 4°C or room temp |
| 25 mL | Wash Solution 1 Concentrate (add 17.5 mL 100% ethanol before use) | 4°C or room temp |
| 45 mL | Wash Solution 2/3 Concentrate (add 36 mL 100% ethanol before use) | 4°C or room temp |
| 25 x 1.3 mL | 2 mL tubes pre-loaded with RNA*later* | room temp |
| 100 mL | Lysis Solution† | room temp |
| 25 | Filter Cartridges | room temp |
| 2 x 25 | Collection Tubes | room temp |
| 2 x 50 mL | Nuclease-free Water | any temp‡ |
| 10 mL | Elution Solution (0.1 mM EDTA) | any temp‡ |

* This reagent contains phenol, which is a poison and an irritant. Use gloves and other personal protection equipment when working with this reagent.

† These components contain guanidinium thiocyanate, a potentially hazardous substance; use with appropriate caution.

‡ Store at –20°C, 4°C, or room temp

## D. Additional Materials Required

**Reagents and materials**

- 100% ethanol, ACS grade or equivalent purity
- 15 mL conical plastic centrifuge tubes, nuclease-free, with volume marks (e.g., P/N AM12500)
- Nuclease-free pipet tips, 200 µL (e.g., P/N AM12655) and 1 mL (e.g., P/N AM12665)
- 5 mL and/or 10 mL pipettes and pipetting tool
- 2 mL nuclease-free microcentrifuge tubes (e.g, P/N AM12425)

**Equipment**

- Microcentrifuge capable of attaining ~15,000 x g
- Table-top (clinical) centrifuge with rotor for 15 mL tubes, capable of attaining ~2,000 x g (e.g., IEC or Straight 8)
- Heat block for 2 mL tubes
- (Optional but recommended) Vacuum manifold and pump (suggested: Athena Technologies vacuum manifold item #4012)
- (Optional but recommended) 5 mL syringe (e.g., BD ref 309603) with plunger removed, to hold Filter Cartridges for vacuum filtration

## E. Related Products Available from Applied Biosystems

| | |
|---|---|
| RNase*Zap*® Solution<br>P/N AM9780, AM9782, AM9784 | RNase*Zap* RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution. |
| RNA 6000 Ladder<br>P/N AM7152 | The RNA 6000 Ladder is a set of 6 RNA transcripts designed for use as reference standards with the RNA 6000 Lab Chip Kits and the Agilent 2100 bioanalyzer. |
| GLOBINclear™-Mouse/Rat Whole Blood Globin Reduction Kit<br>P/N AM1981 | The GLOBINclear-Mouse/Rat Whole Blood Globin Reduction Kit employs a novel, non-enzymatic technology to remove >95% of the globin mRNA from whole blood total RNA samples from mouse or rat. The resulting mRNA is a superior template for RNA amplification and synthesis of labeled cDNA for array analysis. |
| DNA-*free*™ Reagents<br>P/N AM1906 | DNase treatment and removal reagents. This product contains Ambion ultra-high quality RNase-free DNase I and reaction buffer for degrading DNA. It is ideal for removing contaminating DNA from RNA preparations. A novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation is also included. |
| MessageAmp™ aRNA Amplification Kits<br>see our web or print catalog | Ambion offers a full line of MessageAmp 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 Premier and MessageAmp III RNA 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. |
| *mir*Vana™ qRT-PCR Primer Sets and qRT-PCR miRNA Detection Kit<br>P/N AM1558 and see web or print catalog | The *mir*Vana qRT-PCR Primer Sets and qRT-PCR miRNA Detection Kit provide a novel detection system for specific small RNAs. Each qRT-PCR Primer Set includes a primer for reverse transcription and a PCR primer pair optimized for sensitive detection of specific miRNAs by qRT-PCR. *mir*Vana qRT-PCR Primer Sets are available for a comprehensive selection of the human, mouse, and rat miRNAs in the miRNA Registry, as well as for Ambion's exclusive ambi-miRs. The qRT-PCR miRNA Detection Kit provides reagents for amplification of the miRNA targeted by the qRT-PCR Primer Set via traditional endpoint RT-PCR or real-time RT-PCR using SYBR® Green I, and a control primer set. |
| TURBO DNA-*free*™ Kit<br>P/N AM1907 | The TURBO DNA-*free* Kit is ideal for removing contaminating DNA from RNA preparations. The kit employs Ambion® TURBO DNase (patent pending), a specifically engineered hyperactive DNase that exhibits up to 350% greater catalytic efficiency than wild type DNase I. It also includes a novel reagent for removing the DNase without the hassles or hazards of phenol extraction or alcohol precipitation—and without heat inactivation, which can cause RNA degradation. |

# II. Mouse RiboPure-Blood Procedure

## A. Experimental Setup

**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, P/N AM9780).

**Gloves and RNase-free technique**

Wear laboratory gloves at all times during this procedure and change them frequently. Gloves protect you from the reagents, and they protect the RNA from nucleases that are present on skin.

Use RNase-free pipette tips to handle Wash Solutions and Elution Solution, and avoid putting used tips into the kit reagents.

Use the Collection Tubes supplied with the kit; they have been tested for RNase contamination and are certified RNase-free.

**Prepare Wash Solution 1 and Wash Solution 2/3**

a. Add 17.5 mL 100% ethanol to the Wash Solution 1 Concentrate.

b. Add 36 mL 100% ethanol to the Wash Solution 2/3 Concentrate.

c. Mix both solutions well, mark the labels to indicate that the solutions are completed, and store at room temperature.
The completed solutions are called Wash Solution 1 and Wash Solution 2/3 in these instructions.

**Blood samples ≤0.25 mL**

For ≤0.25 mL blood samples, modify the volumes of reagents as described in the procedure.

**(Optional) Set up for vacuum filtration**

**To set up for vacuum filtration**

Remove the plunger of a 5 mL syringe, and attach the barrel to the inlet port of the vacuum manifold.

**Choose vacuum filtration or centrifugation for the glass fiber filtration step**

In step D.1 on page 10, there are different procedures for recovering either total RNA with the small RNA component (e.g., miRNA) or total RNA depleted of small RNAs. The volume of solution to be passed through the Filter Cartridges in the final RNA purification will differ depending on the procedure chosen; consider the preparation volume when deciding whether to use centrifugation or vacuum filtration.

***For recovery of total RNA, including small RNAs such as miRNA***:
the sample volume is 13–15 mL

***For recovery of total RNA depleted of small RNAs such as miRNA:***
the sample volume is 5.5–6.5 mL

- Filtration using a microcentrifuge requires several successive centrifugations (~8 times for samples that are small-RNA–depleted, ~18 times for samples that include small RNAs), but requires no other additional equipment.
- Vacuum filtration allows continuous application of the sample to the Filter Cartridge and is easier when processing larger volumes, but requires a vacuum manifold and pump.

**Assemble and label plasticware for the final RNA purification**

- Briefly inspect the Filter Cartridges before use. Rarely, the glass-fiber filters may become dislodged during shipping. If this is the case, gently push the filter down to the bottom of the cartridge using the wide end of an RNase-free pipet tip.
- For each sample, place a Filter Cartridge into a 2 mL Collection Tube. Label the lid of the resulting Filter Cartridge assembly.
- Label a second Collection Tube for elution and storage of each sample.

**Preheat Elution Solution to 80°C**

At the end of the this procedure, RNA can be eluted in either the Elution Solution provided with the kit or in Nuclease-free Water. Elution Solution is nuclease-free 0.1 mM EDTA, if this could interfere with your application, elute in Nuclease-free Water instead.

In an 80°C heat block, preheat at least 250 µL of Elution Solution or Nuclease-free Water per sample, plus ~5% overage, in a tightly closed nuclease-free 2 mL microcentrifuge tube (not provided).

## B. Sample Collection and Stabilization of RNA

**1. Collect blood samples using standard methods**

Collect mouse or rat blood samples using standard methods (e.g., cardiac puncture, orbital or tail bleed, aspiration from chest cavity after severing hepatic artery, etc.).

- Anticoagulant is not necessary if blood is collected into an airtight container (e.g., a syringe).
- To collect blood into non-airtight containers, use an anticoagulant. EDTA is preferred, because heparin has been reported to inhibit RT-PCR. (Note that our scientists have not observed such inhibition.)

**2. Place ≤0.5 mL blood into 2 mL tubes pre-loaded with RNA*later* and mix**

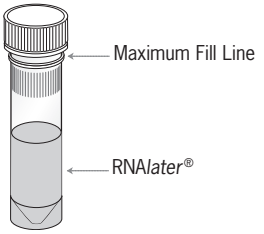



Figure 3. Blood sample fill level in the pre-loaded 2 mL tube.

a. Place ≤0.5 mL blood into the 2 mL tubes pre-loaded with RNA*later* (provided). Figure 3 (left) shows the maximum fill line on the tube for adding the blood sample to the RNA*later*.

**IMPORTANT**

*The amount of RNAlater in the pre-loaded tubes is designed to accommodate no more than 0.5 mL of blood. It is not necessary to determine the exact volume of blood added to the tube, but avoid overfilling the tubes. The ring between the vertical ridges and the collar at the top of the tube marks the maximum fill level when adding blood sample to the tube.*

***For ≤0.25 mL blood:*** Remove and discard 650 µL of the RNA*later* from the pre-loaded 2 mL tube provided. Then add the blood sample to the remaining RNA*later* in the tube.

b. Mix the sample thoroughly by inverting the tube several times.

**3. Store for ≤3 days at room temp/4°C, or long-term at –20°C or below**

- Store ≤3 days at room temperature or 4°C.
- Store long-term at –20°C or below.

## C. Cell Lysis and Initial RNA Isolation

**1. Centrifuge samples for 3 min and remove the supernatant**

***For frozen samples:*** thaw at room temperature. Vortex or shake the tube briefly to disperse its contents.

**a. Centrifuge samples for 3 min at maximum speed (≥15,000 x g) in a microcentrifuge.**

The blood cells and plasma proteins will form a large brown or reddish-brown pellet which may smear upward along the side of the tube. The supernatant is typically turbid, and may be pale pink, brown, or colorless.

**b. Remove and discard the supernatant by aspiration or pouring.**

- Thoroughly remove all of the fluid, even if it is pigmented and/or viscous. Remove even the supernatant directly above the cell pellet, which may be more turbid, and which may contain some white particulate matter. Note: this material is not the "buffy coat" fraction seen in untreated whole blood after centrifugation.
- If the supernatant was poured off, tap the rim of the inverted tube gently against a paper towel to remove all residual fluid.
- Remove any fluid from inside the tube cap.
- The volume of the blood pellet after removing the RNA*later* should be approximately the same as the starting volume of blood added to the tube. If the pellet is significantly larger than this, it is an indication that the separation of RNA*later* from the blood was incomplete.

Proceed with the procedure, but be sure to inspect the sample closely as described in step D.2 on page 11. (See also *Inefficient separation of RNAlater from blood* on page 16.)

**2. Add Lysis Solution and vortex vigorously**

a. Add Lysis Solution to the blood pellet to almost fill the 2 mL tube, leaving sufficient space in the tube to permit thorough mixing.

b. Vortex vigorously to resuspend the blood pellet and lyse the blood cells. Invert the tube and observe to make sure the sample is completely dislodged.

**3. Transfer to a 15 mL tube and add 200 µL 3 M Sodium Acetate**

a. Pour the lysed blood sample into a 15 mL conical tube.

b. Add 200 µL 3 M Sodium Acetate and mix well. Some undissolved clots may be visible at this step.
***For ≤0.25 mL starting blood volume:*** add 100 µL 3 M Sodium Acetate and mix well.

**4. Add Lysis Solution to a total volume of ~3.8 mL and mix thoroughly**

a. Use the volume marks on the 15 mL tube to measure the preparation volume, and determine how much additional Lysis Solution is needed to bring the volume to a total of ~3.8 mL.
***For ≤0.25 mL starting blood volume: plan for a total of ~2 mL.***

b. Rinse the 2 mL tube with a portion of the additional Lysis Solution needed and pool this with the lysate in the 15 mL tube.

c. Add more Lysis Solution, if necessary, to bring the volume to a total of ~3.8 mL.
***For ≤0.25 mL starting blood volume:*** Add Lysis Solution to bring the total volume to ~2 mL.

d. Mix thoroughly by vortexing for ~5 sec. The lysate should now be homogeneous with no undissolved clots. Invert the tube and observe it to be sure the solution is homogenous.

**5. Extract with 1.5 mL Acid Phenol:Chloroform, and recover the aqueous phase**

a. Withdraw 1.5 mL of Acid-Phenol:Chloroform from beneath the overlying layer of aqueous buffer, add it to the cell lysate, close the tube tightly and shake vigorously for 30 sec.
***For ≤0.25 mL starting blood volume:*** Use only 800 µL Acid-Phenol:Chloroform.

b. Store for 5 min at room temperature.

c. Centrifuge for 10 min at ~2,000 x g (~3,200 rpm in a table-top centrifuge) to separate the aqueous and organic phases.
The aqueous (upper) phase should be clear and well-separated, with a thin interface.

d. Recover the aqueous (upper) phase in a fresh 15 mL tube. Discard the organic phase.


NOTE

*The typical volume of the aqueous phase is 3.6–4 mL. Do not recover more than 4.2 mL of aqueous phase or the capacity of the 15 mL tube will be exceeded in the next step if you are using the procedure for recovery of total RNA including small RNAs).*


STOPPING POINT

*If desired, samples may be stored at –20°C for later processing.*
*Samples that will be processed for recovery of total RNA that is depleted of small RNAs may be stored before or after adding the water and ethanol (described below).*
*Store samples that will be processed for recovery of total RNA including small RNAs* ***before*** *adding the water and ethanol to avoid compromising recovery of small RNAs.*
*Warm stored samples to room temp and mix well by vortexing before filtration (described in step 3 on page 11).*

## D. Final RNA Purification

**1. Adjust the volume of the aqueous phase and add ethanol**

There are slightly different instructions to prepare the semi-purified RNA for purification using a glass fiber filter procedure. Follow either step 1a or step 1b below depending on whether you want to obtain total RNA that includes, or is depleted of, the small RNA fraction.

**1a. For recovery of total RNA, including small RNAs such as miRNAs:**

**a. Add 0.6 volume Nuclease-free Water to the aqueous phase.**
For example, if 4 mL of aqueous phase was recovered in step C.5, add 2.4 mL (4 mL x 0.6) of Nuclease-free Water. The solution typically becomes turbid upon addition of the water.

**b. Mix thoroughly by vortexing for ~5 sec.**
The solution usually clears after mixing.

**c. Add 1.2 volumes of 100% ethanol, and vortex for ~10 sec or until the preparation clears.**
Add 1.2 volumes of 100% ethanol relative to the current prep volume. Using the same example, add 7.7 mL of ethanol (1.2 x [4 + 2.4]).

Use the markings on the 15 mL tube to measure the volume of the diluted aqueous phase. The volume of a preparation from a 0.5 mL blood sample after this step should be ~13–15 mL.

**1b. For recovery of total RNA that is depleted of small RNAs:**

**a. Add 0.5 volume 100% ethanol to the aqueous phase.**
For example, if 4 mL of aqueous phase was recovered in step C.5, add 2 mL (4 mL x 0.5) of 100% ethanol. The solution typically becomes turbid upon addition of the 100% ethanol.

**b. Mix thoroughly by vortexing for ~10 sec.**
Observe the solution to ensure that it is homogeneous; if refractive patterns are present, vortex ~5 sec longer.

**2. Check the appearance of the solution**

Rarely, the preparation does not clear, or precipitation or secondary phase separation at the bottom of the tube is observed. If this happens, add Nuclease-free Water in 300 µL increments and vortex until the mixture is homogeneous and essentially clear (slight turbidity is acceptable).

**STOPPING POINT**

*If desired, the prep may be partially filtered as described in the next step and the remainder saved at –20°C for later processing. Warm stored samples to room temp and mix well by vortexing before filtration.*

**3. Pass the sample through a Filter Cartridge**

***For vacuum filtration:***

a. Place a Filter Cartridge in the barrel of a 5 mL syringe attached to an inlet port of the vacuum manifold.

b. Using the vacuum, pass the sample through the filter.

***For microcentrifuge filtration:*** Pass the sample through the Filter Cartridge by centrifuging ~700 µL of sample at a time.

a. Apply ~700 µL of the sample to a Filter Cartridge assembly, close the lid, and centrifuge for ~10 sec in a microcentrifuge set to at least 8,000 x g to pass the liquid through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the same Collection Tube.

b. Load the next ~700 µL of sample onto the filter. Centrifuge as before to pass the sample through the filter and discard the flow-through. Repeat to filter the remaining sample.

**4. Wash filter with 750 µL Wash Solution 1**

***For samples filtered using vacuum filtration:*** Transfer the Filter Cartridge to a labeled Collection Tube.

***For all samples:*** Apply 750 µL Wash Solution 1 to the assembly and centrifuge for ~5–10 sec in a microcentrifuge set to maximum speed to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the same Collection Tube.

**5. Wash filter with 2 x 750 µL Wash Solution 2/3 and dry for 1 min**

a. Apply 750 µL Wash Solution 2/3 to the Filter Cartridge assembly and centrifuge for ~5–10 sec to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the same Collection Tube.

b. Repeat step a with a second 750 µL aliquot of Wash Solution 2/3.

c. Spin the Filter Cartridge assembly for 1 min at maximum speed to remove residual fluid from the filter.

**6. Elute RNA with ~250 µL preheated Elution Solution or Nuclease-free Water**

a. Centrifuge the tube of preheated (80°C) Elution Solution or Nuclease-free Water briefly to remove condensation from the sides and top of the tube.

b. Transfer the Filter Cartridge into a new labeled Collection Tube. Apply ~250 µL preheated Elution Solution or Nuclease-free Water to the center of the filter, and close the cap.
***For ≤0.25 mL starting blood volume:*** use ~150 µL Elution Solution or Nuclease-free Water.


NOTE

*The exact volume of solution used to elute the RNA may be adjusted according to the desired RNA concentration. The RNA can be eluted in a lower volume to increase concentration; however, eluting in less than 150 µL may not provide thorough RNA recovery.*

c. Leave the assembly at room temperature for 1 min.

d. Centrifuge the Filter Cartridge Assembly for 1 min at maximum speed to recover all of the eluted RNA in the Collection Tube. Discard the Filter Cartridge.

e. Store the RNA at –20°C or below.

# III. Assessing RNA Yield and Integrity

## A. RNA Yield

**Expected RNA yield**

The expected RNA yield is 30–55 µg from 0.5 mL of mouse blood. Yield from rat blood is more variable and ranges from 18–40 µg per 0.5 mL of blood.

**Spectrophotometry**

The concentration of an RNA solution can be determined by measuring its absorbance at 260 nm ($A_{260}$). The NanoDrop® 1000A Spectrophotometer is extremely quick and easy to use; measure 1–2 µL of the RNA sample directly, following the manufacturer's instructions.

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).

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

Note 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.

The RiboGreen signal intensity from double stranded DNA is roughly 2-fold more than that for the same mass of RNA; if significant amounts of DNA contaminate an RNA sample, it will lead to overestimation of the RNA concentration.

**Microfluidic analysis: Agilent bioanalyzer**

The Agilent® 2100 bioanalyzer with Caliper's RNA LabChip® Kits provides qualitative and quantitative data for characterizing RNA. This is discussed in the next section, *Microfluidics analysis: Agilent bioanalyzer* on page 13.

## B. RNA Quality

**Microfluidics analysis: Agilent bioanalyzer**

The Agilent® 2100 bioanalyzer with Caliper's RNA LabChip® Kits provides qualitative and quantitative data for characterizing RNA. When used with the Ambion RNA 6000 Ladder (P/N AM7152), this system can quickly provide an 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 mouse or rat blood using this kit and the recommended procedure usually has a 28S to 18S rRNA ratio of >0.8.

Using the bioanalyzer, the RIN (RNA Integrity Number) can be determined to further evaluate RNA integrity. A new metric developed by Agilent, the RIN algorithm analyzes information from both major rRNA bands, as well as information contained outside the rRNA peaks (i.e., potential degradation products) to provide a more complete picture of RNA integrity. Search for "RIN" at Agilent's website for information:

www.chem.agilent.com

Figure 4 shows a representative bioanalyzer profile of RNA prepared using the Mouse RiboPure-Blood Kit. Note that recovery of small RNA can be verified on the bioanalyzer.

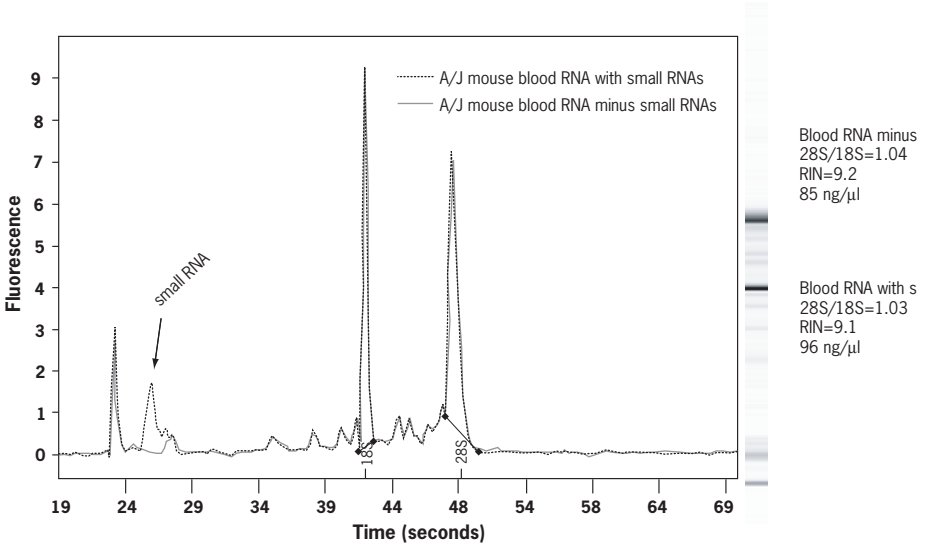



Figure 4. Bioanalyzer Profile of RNA Prepared with the Mouse RiboPure™-Blood RNA Isolation Kit

The Mouse RiboPure-Blood Kit was used to isolate RNA from 0.5 mL blood from a male A/J strain mouse. The aqueous phase recovered after the phenol/chloroform extraction step was split into two aliquots. One was processed for recovery of total RNA including miRNA and other small RNAs, and the other for recovery of total RNA which is depleted of small RNAs.

### 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 1.9–2.2. Lower ratios can indicate protein or other contamination in the sample. However, RNA with absorbance ratios outside of this range may still function well for downstream applications.

### Agarose gel analysis

RNA yields and integrity can also be assessed by traditional denaturing gel electrophoresis. A protocol is provided on our website at the following address:

www.ambion.com/techlib/append/supp/rna_gel.html

We recommend loading ~15 µL of RNA obtained using this procedure. High quality RNA will show sharp prominent bands representing the 28S and 18S rRNA. Diffuse, fast-migrating bands of low molecular weight RNAs (e.g., 5S rRNA and tRNA) may also be visible. If genomic DNA is present, it is visible as high molecular weight material that may remain in the wells of the gel, or may migrate as a distinct band or as a smear.

# IV. Troubleshooting

## A. Low RNA Yield/ RNA Degradation

**Inefficient separation of RNA*later* from blood**

The RNA*later*® Solution(1.3 mL) provided in the 2 mL tubes is expected to separate from the blood after the centrifugation in step II.C.1 on page 8. The amount of RNA*later* in the pre-loaded tubes is designed to accommodate a maximum of 0.5 mL of blood. If more than 0.5 mL of blood is used, incomplete separation of blood from the RNA*later* may result, and the blood pellet in step II.C.1 will be significantly larger than the volume of the original blood sample.

**Suggestions to solve or avoid the problem**

- In subsequent preparations, limit the blood sample volume to no more than 0.5 mL.
- To obtain RNA from samples in which poor separation of the sedimented blood from the RNA*later* is seen in step II.C.1, follow these recommendations:

1. Respin the tube for ~2 min at maximum speed and remove all residual RNA*later*, typically ~100 µL.
2. Continue with the procedure and observe the preparation closely as described in step II.D.2 on page 11 to see if the solution clears as expected. If it does not clear, you may see one of the following:
   - Turbid solution
   - An insoluble precipitate forms
   - Small droplets are visible that sink to the bottom of the tube (secondary phase separation).
3. If your preparation resembles any of the descriptions above, add 300 µL Nuclease-free Water and mix thoroughly (i.e., vortex vigorously for 5–10 sec). Repeat this process, adding Nuclease-free Water in 300 µL increments, and mixing thoroughly after each addition, until the preparation is homogeneous and essentially clear (slight turbidity is OK).
   Usually only one or two additions of 300 µL water are required.

**Suboptimal storage of blood samples**

A gradual decrease in RNA yield and quality is seen in samples stored in RNA*later* at ambient temperature for more than 3 days.

**Other suggestions for troubleshooting low yield/ degradation**

The following suggestions may improve RNA recovery/assay results.

- Use a smaller volume of blood (0.3–0.4 mL instead of 0.5 mL).
- Store samples stabilized in RNA*later* at –20°C instead of at room temperature, and/or shorten the storage time in RNA*later*.

- ***For RT-PCR assays,*** some degradation of RNA can be tolerated, if small amplicons are used. We recommend using PCR primers that amplify an amplicon of ~100–300 bp in such situations.

## B. RNA Contains Residual Genomic DNA

**Treat the eluted RNA with DNase I**

The eluted RNA can be treated with DNase I as described in *V.A. (Optional) DNase I Treatment* on page 18.

**Use fewer PCR cycles and/or less RNA in the RT-PCR**

Genomic DNA may be amplified in RT-PCR samples that are subjected to >30 PCR cycles and/or when an unusually large amount of RNA (>1 µg) is used in the RT-PCR.

# V. Appendix

## A. (Optional) DNase I Treatment

The eluted RNA can be treated with DNase I to remove trace levels of genomic DNA.

- Use either the Ambion DNA-*free* Kit (P/N AM1906), which includes Ambion ultra-high quality RNase-free DNase I or the TURBO DNA-*free* Kit (P/N AM1907), supplied with our catalytically superior TURBO DNase™. Both products include a novel reagent for removing the DNase without phenol treatment or heat inactivation.
- Alternatively, treat with ~2–8 units of RNase-free DNase I (P/N AM2222, AM2224) in 1X DNase buffer for ~15–30 min. at 37°C. Inactivate the DNase by phenol extraction and alcohol precipitation.

## B. Quality Control

**Functional testing**

The kit is functionally tested by isolating RNA from mouse whole blood using the procedure described in this protocol. RNA yield is determined by absorbance measurements using the NanoDrop Spectrophotometer. RNA integrity is evaluated using 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 supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

**Exonuclease activity**
Meets or exceeds specification when a sample is incubated with labeled double-stranded DNA, followed by PAGE analysis.

## C. Safety Information

**Chemical safety guidelines**

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety 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.

### About MSDSs

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

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

### Obtaining the MSDS

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

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

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