PROTOCOL

ambion® | RNA
by life technologies™

*mir*Vana™ PARIS™ Kit

life technologies™

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*mir*Vana™ PARIS™ Kit

(Part Number AM1556)

Protocol

I. Introduction

IMPORTANT

Before using this product, read and understand the "Safety Information" in the appendix in this document.

A. Product Description

The *mir*Vana™ PARIS™ Kit was designed for isolation of both protein and RNA suitable for studies of small RNA expression, processing or function. The kit employs the Ambion® PARIS (Protein And RNA Isolation System) technology to recover both native protein and RNA from the same experimental sample. For optimal recovery of small RNAs, an organic extraction followed by immobilization of RNA on glass-fiber filters is used to purify either total RNA, or an RNA fraction enriched for small RNA species.

The *mir*Vana PARIS Kit can be used to isolate both native protein and small RNA. To recover native protein from cells or tissues, samples are first homogenized in a buffer containing a nonionic detergent (Figure 1 on page 4). A fraction of this lysate can be used directly for common applications such as enzymatic assays, immunoprecipitation, gel shift assays, two-dimensional gel electrophoresis, and Western blotting. RNA can be isolated from the remainder of the lysate using a procedure that combines the advantages of organic extraction and solid-phase extraction, while avoiding the disadvantages of both. High yields of ultra-pure RNA can be prepared in about 30 min. The high quality RNA recovered can be used in any application, including RT-PCR, RNA amplification, microarray analyses, solution hybridization assays, and blot hybridization.

B. Background

Noncoding small RNAs such as transfer RNAs (tRNA), 5S and 5.8S ribosomal RNAs (rRNA), small nucleolar RNAs (snoRNA), and small nuclear RNAs (snRNA) play critical roles in many biological processes. In the past few years, interest in the identification, detection, and use of small RNA molecules has rapidly expanded. This interest is the result of two related lines of research. In one, small double-stranded RNAs (dsRNAs) called small interfering RNAs (siRNAs) are used to silence the expression of specific genes at the post-transcriptional level by a pathway known as RNA interference (RNAi). In the other, numerous small regulatory RNA molecules, referred to as microRNAs (miRNAs), have been shown to regulate target gene expression at the translational level. Both miRNAs and siRNAs range between 15–30 nucleotides in length.

In addition to isolating small RNAs and monitoring their expression, miRNA and siRNA studies also often require analysis of protein expression levels. In RNAi experiments for example, siRNA tools are used to direct the degradation of specific messenger RNAs (mRNAs), resulting in target knockdown at both the mRNA and the protein level. In contrast, miRNAs often act as repressor of translation, affecting only protein expression levels.

Traditional RNA isolation methods are not well suited for isolation of small RNAs

Variations of two methods have historically been used to isolate RNA from biological samples: chemical extraction or immobilization on glass (silica)—often referred to as solid-phase extraction. Chemical extraction methods usually use highly concentrated chaotropic salts in conjunction with acidic phenol or phenol-chloroform solutions to inactivate RNases and purify RNA from other biomolecules. These methods provide very pure preparations of RNA; however, the RNA must typically be desalted and concentrated by alcohol precipitation. Routine alcohol precipitation of total RNA does not quantitatively recover small nucleic acid molecules, making it ill-suited for the preparation of very small RNAs.

The second method, solid-phase extraction, relies on high salt or salt and alcohol to decrease the affinity of RNA for water and to increase its affinity for the solid support used. The use of glass as a solid support has been shown to work for large RNAs (Boom et al. 1990). The conditions routinely used for silica-based solid-phase purification of RNA, however, do not effectively recover small RNAs.

C. Summary of the *mir*Vana PARIS Kit Procedure

Sample disruption and organic extraction

The first step of the *mir*Vana PARIS procedure is to disrupt samples in Cell Disruption Buffer. A portion of the resulting lysate can be reserved for protein analysis, and the remainder can be processed for RNA isolation. For RNA isolation, the lysate is mixed with the 2X Denaturing Solution and subjected to Acid-Phenol:Chloroform extraction which provides a robust front-end RNA purification that also removes most DNA (Chomczynski, 1987).

Final RNA purification over glass-fiber filter

There are separate procedures for purification of either total RNA—including very small RNA species—or for purifying RNA that is highly enriched for small RNA species and contains very little RNA larger than about 200 bases. Figures 2 and 3 starting on page 4 show representative examples of total, depleted, and enriched RNA fractions prepared with the *mir*Vana PARIS Kit.

IMPORTANT

To isolate RNA for miRNA expression profiling using miRNA arrays, we recommend following the procedure for total RNA isolation (not the enrichment procedure for small RNAs). This makes it possible to critically evaluate the quality of the RNA to verify that it is suitable for array analysis and to quantitate the RNA. The miRNA population should then be further purified (e.g., with the flashPAGE™ system) before labeling samples for miRNA array analysis.

Final purification of total RNA

The procedure for isolation of total RNA is similar to routine glass-fiber binding protocols. Ethanol is added to samples, and they are passed through a Filter Cartridge containing a glass-fiber filter which immobilizes the RNA. The filter is then washed a few times, and finally the RNA is eluted with a low ionic-strength solution.

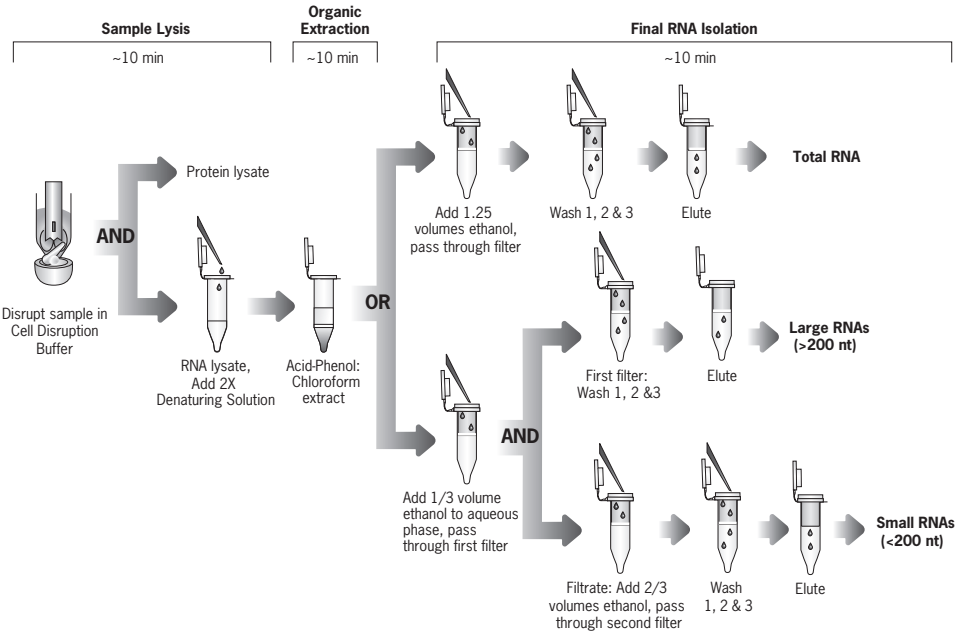
Final purification of RNA enriched for small RNAs

To isolate RNA that is highly enriched for small RNA species, 100% ethanol is added to bring the samples to 25% ethanol. When this lysate/ethanol mixture is passed through a glass-fiber filter, large RNAs are immobilized, and the small RNA species are collected in the filtrate. The ethanol concentration of the filtrate is then increased to 55%, and it is passed through a second glass-fiber filter where the small RNAs become immobilized. This RNA is washed a few times, and eluted in a low ionic strength solution. Using this novel approach consisting of two sequential filtrations with different ethanol concentrations, an RNA fraction highly enriched in RNA species <200 nt can be obtained. Note that the large RNA species (>200 nt) can be recovered from the first filter if necessary.

D. Reagents Provided with the Kit and Storage

This kit contains reagents for 40 isolations of protein and total RNA, or 20 isolations of protein, and separate large and small RNA fractions.

Figure 1. Overview of the *mir*Vana PARIS Procedure.



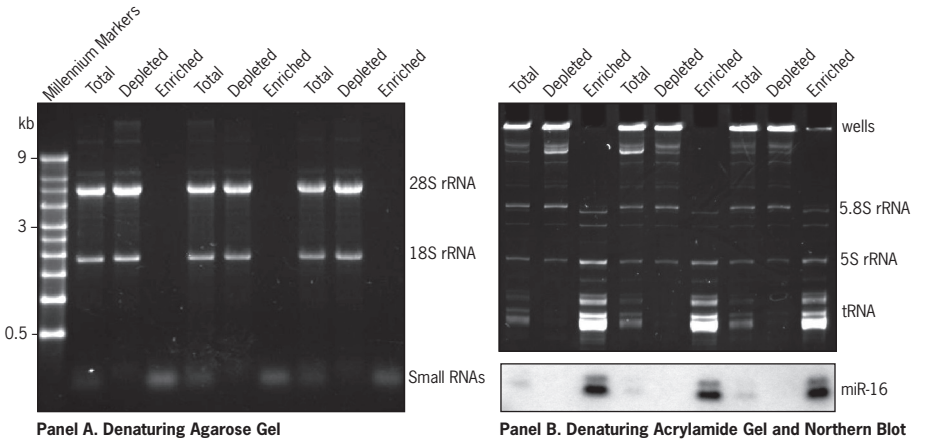


Figure 2. RNA Isolated from Cultured Cells Using the *mir*Vana™ PARIS™ Kit.

Total RNA or fractions depleted or enriched in small RNA were isolated in triplicate from 1 x 10^6 HeLa cells with the *mir*Vana PARIS Kit. (A) 1 µg of each fraction was resolved on a 1.2% denaturing glyoxal agarose gel and stained with ethidium bromide. (B) 1 µg of each fraction was resolved on a 15% denaturing acrylamide gel and stained with ethidium bromide. miR-16 miRNA was detected by Northern blot (see procedure on page 25) with an antisense RNA probe labeled and purified with the *mir*Vana Probe & Marker Kit (P/N AM1554).

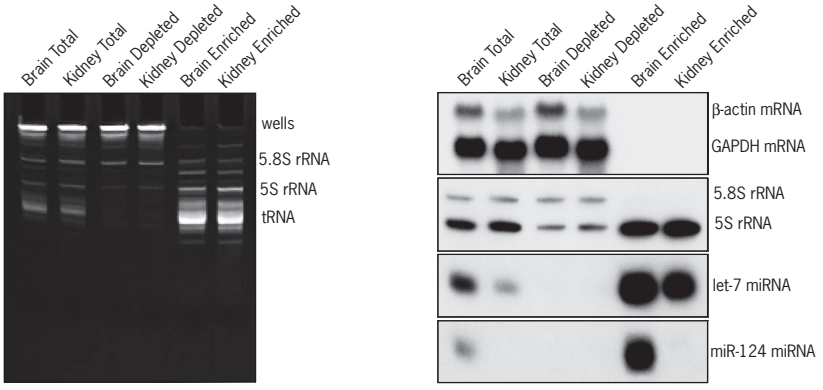


Figure 3. RNA Isolation from Mouse Tissues.

Total RNA or fractions depleted or enriched in small RNA were isolated from RNA*later*-treated mouse brain or kidney (~50 mg). (A) 1 µg of each fraction was resolved on a 15% denaturing acrylamide gel and stained with ethidium bromide. (B) 1 µg of total RNA was used for detection of the indicated RNA species. 5.8S and 5S rRNAs and let-7 miRNA were detected by Northern blot using DNA probes (see procedure on page 25). miR-124 miRNA was detected by solution hybridization with the *mir*Vana miRNA Detection Kit (P/N AM1552) and an antisense RNA probe. Small RNA probes were 5' labeled and purified with the *mir*Vana Probe & Marker Kit. β-actin and GAPDH mRNAs were analyzed using the NorthernMax®-Gly Kit (P/N AM1946).

Amount	Component	Storage
30 mL	miRNA Wash Solution 1 Add 21 mL 100% ethanol before use	room temp*
50 mL	Wash Solution 2/3 Add 40 mL 100% ethanol before use	room temp*
80	Collection Tubes	room temp
40	Filter Cartridges	room temp
25 mL	Cell Disruption Buffer	4°C
25 mL	2X Denaturing Solution Add 375 µL 2-mercaptoethanol before use	4°C
2 x 25 mL	Acid-Phenol:Chloroform	4°C
5 mL	Elution Solution	any temp†

* 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, 4°C or room temp

Note that the kit is shipped at room temperature which will not affect its stability.

E. Materials Not Provided with the Kit

Reagents

- 2-mercaptoethanol (14.3 M)
- 100% ethanol, ACS grade or better
- Phosphate buffered saline (PBS)

Equipment and supplies

- Heat block set to 95–100°C
- Microcentrifuge capable of at least 10,000 x g
- (optional) vacuum manifold: to pull solutions through the Filter Cartridges
- RNase-free 1.5 mL or 0.5 mL polypropylene microfuge tubes, adjustable pipettors and RNase-free tips
- Tissue homogenizer (for solid tissue samples): motorized rotor-stator homogenizers work best, but for soft tissues and very small samples manual homogenizers may be sufficient – see section I.D starting on page 10 for more information on sample disruption.

F. Related Products

RNA*later*® Solution P/N AM7020, AM7021	RNA*later* Tissue Collection: RNA Stabilization Solution is an aqueous sample collection solution that stabilizes and protects cellular RNA in intact, unfrozen tissue and cell samples. RNA*later* Solution eliminates the need to immediately process samples or to freeze samples in liquid nitrogen. Samples can be submerged in RNA*later* Solution for storage at RT, 4°C, or –20°C without jeopardizing the quality or quantity of RNA that can be obtained.
RNase*Zap*® Solution P/N AM9780, AM9782, AM9784	RNase*Zap* RNase Decontamination Solution is simply sprayed, poured, or wiped onto surfaces to instantly inactivate RNases. Rinsing twice with distilled water will eliminate all traces of RNase and RNase*Zap* Solution.
TURBO DNA-*free*™ Kit P/N AM1907	The TURBO DNA-*free* Kit is ideal for removing contaminating DNA from RNA preparations. The kit employs Ambion® TURBO DNase, 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.
*mir*Vana™ miRNA Probe Construction Kit P/N AM1550	This kit is designed to produce short (<100 nt) labeled RNA transcripts for use in hybridization assays to detect small RNAs, including miRNA and siRNA. The kit supplies reagents for both transcription template preparation and RNA probe synthesis. Radiolabeled probes made with the kit are ideal for use with the *mir*Vana miRNA Detection Kit.

*mir*Vana™ miRNA Detection Kit P/N AM1552	The *mir*Vana miRNA Detection Kit provides an extremely sensitive solution hybridization assay capable of detecting attomole amounts of RNA. In addition, it can be used to simultaneously detect several small RNAs such as miRNA and siRNA, or both small RNA and long RNA species in the same sample. For a complete solution for small RNA analysis, use this kit in conjunction with the *mir*Vana miRNA Probe Construction Kit and/or the *mir*Vana Probe & Marker Kit.
*mir*Vana™ Probe & Marker Kit P/N AM1554	The *mir*Vana Probe & Marker Kit is an end labeling kit designed for making short radiolabeled probes, and low molecular weight markers for studies involving microRNAs. It can be used with synthetic RNA or DNA oligonucleotides to prepare labeled probes, and the kit also provides reagents to prepare small radiolabeled RNA size markers (Decade™ Markers), and single-nucleotide RNA ladders. Rapid cleanup reagents are included to prepare the reaction products for various downstream application.
Decade™ Markers P/N AM7778	The Decade Marker System is a set of reagents to prepare radiolabeled low molecular weight RNA markers: from 10–100 nt in 10 nt increments. The user supplies only [γ-^{32}P]ATP to end label a single, gel purified RNA transcript which is then cleaved into the 10 molecular weight markers in a simple 5 minute reaction.
PAGE Reagents	We offer high-quality reagents for polyacrylamide gel electrophoresis (PAGE), including acrylamide solutions for RNA, DNA and protein gels, ultra-pure molecular biology grade urea, and premixed acrylamide/urea solutions for simple preparation of denaturing gels.

II. *mir*Vana PARIS Procedure

A. Solution Preparation

Add 375 µL 2-mercaptoethanol to the 2X Denaturing Solution

Add 375 µL of 2-mercaptoethanol to the bottle labeled 2X Denaturing Solution. Mix well. Place a check mark in the box on the label to indicate that the 2-mercaptoethanol has been added.


NOTE

The 2X Denaturing Solution may solidify at 4°C. Before use, warm the solution at 37°C with occasional agitation for 5–10 min, or until it is completely in solution. To avoid solidification, you may store the 2X Denaturing Solution at room temp for up to 1 month if desired.

Add 21 mL 100% ethanol to miRNA Wash Solution 1

Add 21 mL of ACS grade 100% ethanol to the bottle labeled miRNA Wash Solution 1. Mix well. Place a check mark in the box on the label to indicate that the ethanol has been added.

Add 40 mL 100% ethanol to Wash Solution 2/3

Add 40 mL of ACS grade 100% ethanol to the bottle labeled Wash Solution 2/3. Mix well. Place a check mark in the box on the label to indicate that the ethanol has been added.


NOTE

A precipitate may form in the Wash Solution 2/3 bottle over the next several days as excess EDTA falls out of solution. Simply leave these crystals in the bottle when removing Wash Solution for use.

B. Equipment Preparation

a. 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*®).

b. Gloves and RNase-free technique

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

Use RNase-free pipette tips to handle the kit reagents.

c. Preparing equipment

The equipment used for tissue disruption/homogenization should be washed well with detergent and rinsed thoroughly. If samples will be ground in a mortar and pestle, prechill the equipment in dry ice or liquid nitrogen.

C. Sample Type and Amount

IMPORTANT

Native protein for functional or enzymatic assays can be recovered only from fresh samples.

Sample type

Tissue or cultured cell samples

This procedure is designed for small scale RNA and protein isolation from animal tissue or cultured eukaryotic cells. The cell disruption procedure can be used with fresh or frozen cultured mammalian cells, or cells stored in RNA*later* or in RNA*later*-ICE. It can also be used with fresh, frozen, or RNA*later*-stored animal tissues that do not contain high levels of RNase. Following is a list of tissues known to be compatible or incompatible with the *mir*Vana PARIS procedure:

Compatible	Incompatible
brain	pancreas
heart	spleen
kidney	
liver	
thymus	

Liquid samples (RNA only)

Because the Denaturing Solution is supplied at a 2X concentration, the kit can also be used to recover total RNA or small RNAs (but not protein) from liquid samples such as fresh blood, plasma, tissue homogenate, and nuclear or cytoplasmic extracts. For liquid samples, start the procedure at section II.E on page 13.

Sample amount

- Samples containing 10^2–10^7 cultured eukaryotic cells or 0.5–100 mg of tissue can be processed per prep.
- Liquid samples as large as 625 µL can be processed for RNA isolation only.

D. Sample Disruption

1. Collect 10^2–10^7 cells or 0.5–100 mg tissue Wash cells in cold PBS

In this procedure, sample size is limited by the amount of lysate that can be passed through a Filter Cartridge without clogging. These instructions are written to prepare the amount of lysate that can be processed on a single Filter Cartridge.

Cultured mammalian cells

Ideally cells in culture should be processed fresh (i.e. not frozen). If you need to store cells before RNA isolation, they can be stored in RNA*later*, or they can be pelleted, snap-frozen in liquid nitrogen, and stored colder than –70°C.

Suspension cells: Count the cells, then pellet the volume of cell culture containing 10^2–10^7 cells at low speed (appropriate for your cells), and discard the culture medium. Wash the cells by resuspending in ~1 mL PBS, and repelleting. Place the cells on ice.

Adherent cells: do one of the following

- Aspirate and discard the culture medium, and rinse with PBS. Place the culture plate on ice.
- Trypsinize cells to detach them and count. Then inactivate the trypsin, pellet the cells, and discard the supernatant (following the method employed in your lab for the cell type). Wash the cells by gently resuspending in ~1 mL PBS, and pelleting at low speed. Place the cells on ice.

Tissue samples

For good yield of intact RNA, it is very important to obtain tissue quickly and to limit the time between obtaining tissue samples and inactivating RNases in step "c" below.

a. Obtain tissue and remove as much extraneous material as possible, for example remove adipose tissue from heart, and remove gall bladder from liver. The tissue can be perfused with cold PBS if desired to eliminate some of the red blood cells.

b. If necessary, quickly cut the tissue into pieces small enough for either storage or disruption. Weigh the tissue sample (this can be done later for samples that will be stored in RNA*later*).

c. Inactive RNases by one of the following methods:
   - Drop the sample into RNA*later*—tissue must be cut to ≤0.5 cm in at least one dimension.
   - Disrupt the sample in Cell Disruption Buffer as described in step D.2. *Fresh (unfrozen) tissue* on page 11.

- Freeze the sample in liquid nitrogen—tissue pieces must be small enough to freeze in a few seconds. When the liquid nitrogen stops churning, it indicates that the tissue is completely frozen. Once frozen, remove the tissue from the liquid nitrogen and store it in an airtight container below –70°C.

2. Disrupt samples in 100–625 µL ice-cold Cell Disruption Buffer

The exact volume of Cell Disruption Buffer is not critical. If your sample amount is near the suggested maximum (10^7 cells or 100 mg tissue), then use near the maximum recommended amount of Cell Disruption Buffer, and conversely, use a low end amount of buffer for relatively small samples. The Cell Disruption Buffer volume may also be adjusted according to the amount of lysate you want for protein analysis and its desired concentration. If required for specific applications, protease, phosphatase and/or RNase inhibitors can be added to an aliquot of Cell Disruption Buffer immediately before use.


NOTE

Our website has comprehensive information on tissue disruption. Go to www.invitrogen.com/ambion, then type "disruption" into the search engine.

Cultured cells

a. Remove the PBS wash or the RNA*later*, and add 100–625 µL of ***ice-cold*** Cell Disruption Buffer to the cells. Use at least 300 µL for ≥10^6 cells.
   The cells will begin to lyse immediately upon exposure to the Cell Disruption Buffer. For adherent cells lysed directly in the culture plate, collect the lysate with a rubber spatula, and transfer it to a tube.

b. Vortex or pipet vigorously to completely lyse the cells and to obtain a homogenous lysate. Keep samples cold during lysis.
   Large frozen cell pellets (i.e. containing more than about 10^7 cells) may need to be ground to a powder as described for frozen tissue samples to isolate high quality RNA.

Solid tissue stored in Ambion® RNA*later*®, or transitioned to –20°C in RNA*later*-ICE

Samples in RNA*later* and RNA*later*-ICE can usually be homogenized by following the instructions for fresh tissue (below). Extremely tough/fibrous tissues in RNA*later* may need to be frozen and pulverized according to the instructions for frozen tissue in order to achieve good cell disruption.

Blot excess RNA*later* from samples, and weigh them before following the instructions for fresh tissue below.

Fresh (unfrozen) tissue

a. Blot tissue on absorbent paper to remove excess moisture or RNA*later*. Measure or estimate the weight of the sample.

b. Aliquot 100–625 µL (6–8 volumes per tissue mass) of ***ice-cold*** Cell Disruption Buffer into a homogenization vessel on ice.

IMPORTANT
Keep the sample cold during disruption.

c. Thoroughly disrupt the tissue in Cell Disruption Buffer using a motorized rotor-stator homogenizer (e.g. Polytron). Manual homogenizers can be used for small pieces (less than ~10 mg) of soft tissue.

Frozen tissue, and extremely hard tissues
(Frozen tissue transitioned to –20°C in RNA*later*-ICE: process as for fresh tissue). Once the tissue has been removed from –70°C, it is important that it be processed immediately without allowing any thawing to occur. This is necessary because as cells thaw, ice crystals rupture both interior and exterior cellular compartments, releasing RNase.

a. Blot tissue on absorbent paper to remove excess moisture or RNA*later*. Measure or estimate the weight of the sample.

b. Place 100–625 µL (6–8 volumes per tissue mass) of ***ice-cold*** Cell Disruption Buffer into a plastic weigh boat on ice.
(It is much easier to transfer the frozen powder to a weigh boat than to a *tube* of Cell Disruption Buffer.)

c. Grind frozen tissue to a powder with liquid nitrogen in a pre-chilled mortar and pestle sitting in a bed of dry ice.

d. Using a pre-chilled metal spatula, scrape the powdered tissue into the Cell Disruption Buffer, and mix rapidly.

e. Working quickly, bend the weigh boat to form a spout, and transfer the mixture to a vessel for homogenization. Process the mixture to homogeneity using a motorized rotor-stator homogenizer (e.g. Polytron).

3. Split the lysate for RNA isolation and protein analysis

a. Immediately proceed to step II.E.1 on page 13 with the portion of the lysate that will be used for RNA isolation.
Once the lysate is homogenized, immediately mix the portion that will be used for RNA isolation with an equal volume of 2X Denaturing Solution at room temp (see step II.E.1 on page 13). The high concentration of guanidinium thiocyanate in the 2X Denaturing Solution will prevent RNA degradation by cellular ribonucleases.

b. Place the portion of lysate that will be used for protein analysis on ice for 5–10 min. Then store it, or use it for protein analysis.
Incubate the lysate (for protein analysis) on ice for 5–10 min to ensure complete cell disruption before processing the sample for protein analysis. If desired, samples can be clarified by centrifugation at

4°C for 1–2 min at top speed in a microcentrifuge. Protein lysates may be very viscous, if this is the case, reduce viscosity by sonicating the lysate, or by passing it through a syringe needle several times.

After this brief incubation on ice, the protein lysate can be used directly for protein analysis. See section *III.B. Evaluating Yield and Quality of Protein Samples* on page 19 for more information.

E. Organic Extraction

IMPORTANT

The 2X Denaturing Solution may precipitate if kept on ice or at 4°C. Pre-warm the solution to 37°C in advance if required.

1. Mix the lysate with an equal volume of 2X Denaturing Solution and incubate on ice for 5 min

a. Transfer the lysate that will be used for RNA isolation to a tube containing an equal amount of 2X Denaturing Solution at room temp. Immediately mix thoroughly.

b. Incubate the mixture on ice for 5 min.

For liquid samples:

To isolate RNA from liquid samples, mix up to 625 µL of sample with an equal volume of 2X Denaturing Solution at room temp. Then follow the standard procedure described below and in section II.F. If the sample volume is less than 100 µL, add Cell Disruption Buffer to bring the sample to ≥100 µL, and immediately mix with an equal volume of 2X Denaturing Solution.

2. Extract with an equal volume of Acid-Phenol:Chloroform

a. Add a volume of Acid-Phenol:Chloroform equal to the total volume of the sample lysate plus the 2X Denaturing Solution. (Be sure to withdraw the bottom phase containing Acid-Phenol:Chloroform, not the aqueous buffer that lies on top of the mixture.)

For example, if the initial sample lysate volume was 200 µL and it was mixed with 200 µL of 2X Denaturing Solution in step 1, add 400 µL Acid-Phenol:Chloroform.

IMPORTANT

Be sure to withdraw the bottom phase containing Acid-Phenol:Chloroform, not the aqueous buffer that lies on top of the mixture.

b. Vortex for 30–60 sec to mix.

c. Centrifuge for 5 min at maximum speed (≥10,000 x g) at room temp to separate the mixture into aqueous and organic phases. After centrifugation, the interphase should be compact; if it is not, repeat the centrifugation.

3. Recover the aqueous phase; and transfer it to a fresh tube

Carefully remove the aqueous (upper) phase without disturbing the lower phase or the interphase, and transfer it to a fresh tube. Note the volume recovered.

F. Final RNA Isolation

Choice of eluent

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

Pre-heat eluent to 95°C

Preheat Elution Solution or nuclease-free water to 95°C for use in eluting the RNA from the filter at the end of the procedure.

100% ethanol must be at room temperature

If the 100% ethanol you plan to use for this procedure is stored cold, warm it to room temperature before starting the Final RNA Isolation.

Separate procedures for large and small RNA isolation

Separate procedures are provided for isolating total RNA (section II.F.I below), or for isolating RNA that is highly enriched for RNAs smaller than ~200 bases (section II.F.II on page 15). Choose the procedure that is better suited to your needs.

IMPORTANT

To isolate RNA for miRNA expression profiling using miRNA arrays, we recommend following the procedure for total RNA isolation (not the enrichment procedure for small RNAs). This makes it possible to critically evaluate the quality of the RNA to verify that it is suitable for array analysis and to quantitate the RNA. The miRNA population should then be further purified (e.g., with the flashPAGE™ system) before labeling samples for miRNA array analysis.

Spin Filter Cartridges at 10,000 x g

Centrifuge Filter Cartridges at RCF 10,000 x g. Spinning harder than this may damage the filters.

F.I. Total RNA Isolation Procedure

1. Add 1.25 volumes 100% ethanol to the aqueous phase, and mix thoroughly

Add 1.25 volumes of room temperature 100% ethanol to the aqueous phase from step E.3 (e.g. if 300 µL was recovered, add 375 µL ethanol) and mix thoroughly.

2. Pass the lysate/ethanol mixture through a Filter Cartridge

a. For each sample, place a Filter Cartridge into one of the Collection Tubes.

b. Pipet the lysate/ethanol mixture onto the Filter Cartridge. Up to 700 µL can be applied to a Filter Cartridge at a time, for samples larger than this, apply the mixture in successive applications to the same filter.

c. Centrifuge for ~30 sec or until the mixture has passed through the filter.
Alternatively, vacuum pressure may be used to pass samples through the filter.

d. Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter. Save the Collection Tube for the washing steps.

3. Wash the filter with 700 µL miRNA Wash Solution 1

Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~15 sec or use a vacuum to pull the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

4. Wash the filter twice with 500 µL Wash Solution 2/3

a. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.

b. Repeat with a second 500 µL of Wash Solution 2/3.

c. After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min at to remove residual fluid from the filter.

5. Elute RNA with 100 µL 95°C Elution Solution or Nuclease-free Water

Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µL of preheated (95°C) Elution Solution or nuclease-free water to the center of the filter, and close the cap. Centrifuge for ~30 sec to recover the RNA.

Collect the eluate (which contains the RNA) and store it at –20°C or colder.

F.II. Enrichment Procedure for Small RNAs

This variation of a traditional glass-fiber filter RNA purification yields RNA that is significantly enriched for small RNAs. This enrichment is accomplished by first immobilizing large RNAs on the filter with a relatively low ethanol concentration and collecting the flow-through containing mostly small RNA species. More ethanol is then added to this flow-through, and the mixture is passed through a second glass filter where the small RNAs are immobilized. Both filters are then washed a few times, and the large- (first filter) or small-RNA (second filter) enriched fractions are eluted.

1. Add 1/3 volume 100% ethanol to the aqueous phase, and mix thoroughly

Add 1/3 volume of 100% ethanol to the aqueous phase recovered from step E.3 (e.g. if 300 µL was recovered, add 100 µL 100% ethanol) and mix thoroughly.

2. Pass the lysate/ethanol mixture through a Filter Cartridge, and collect the filtrate

a. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied.

b. Pipet the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. Up to 700 µL can be applied to a Filter Cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.

c. Centrifuge for ~30 sec or until the mixture has passed through the filter.
Alternatively, vacuum pressure can be used to pull samples through the filter.

d. ***Collect the filtrate (or flow-through).*** If the lysate/ethanol mixture is >700 µL, transfer the filtrate to a fresh tube, and repeat until all of the lysate/ethanol mixture is through the filter. Pool the collected filtrates if multiple passes were done, and measure the total volume of the filtrate.


NOTE

At this point, the filter contains an RNA fraction that is depleted of small RNAs. This fraction can be recovered if desired by treating the filter as described in steps F.II.5–7.

3. Add 2/3 volume 100% ethanol to the filtrate, and mix thoroughly

Add 2/3 volume room temperature 100% ethanol to filtrate (flow-through). For example, if 400 µL of filtrate is recovered, add 266 µL 100% ethanol. Mix thoroughly.

4. Pass the filtrate/ethanol mixture through a second Filter Cartridge, and discard the flow-through

Pass the filtrate/ethanol mixture through a second Filter Cartridge as in step 2 above, but this time discard the flow-through. Reuse the Collection Tube for the washing steps.

5. Wash the filter with 700 µL miRNA Wash Solution 1

Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~15 sec or use vacuum pressure to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

6. Wash the filter twice with 500 µL Wash Solution 2/3

a. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.

b. Repeat with a second 500 µL of Wash Solution 2/3.

c. After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and centrifuge the assembly for 1 min to remove residual fluid from the filter.

7. Elute small RNA with 100 µL 95°C Elution Solution or Nuclease-free Water

Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µL of preheated (95°C) Elution Solution or nuclease-free water to the center of the filter, and close the cap. Centrifuge for ~30 sec to recover the RNA.

Collect the eluate (which contains the RNA) and store it at –20°C or colder.

III. Evaluating Yield and Quality

A. Evaluating Yield and Quality of RNA Samples

1. RNA concentration

The concentration and purity of an RNA solution can be determined by measuring its absorbance at 260 and 280 nm. We highly recommend using the NanoDrop 1000A Spectrophotometer for spectrophotometer readings. The NanoDrop is extremely quick and easy to use because no dilutions and no cuvettes are needed. Just measure 1.5 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. Include a measurement at 280 nm as well to get a rough idea of RNA purity (see section A.3 below). An A_{260} of 1 is equivalent to 40 µg RNA/mL. Find the concentration in µg/mL by multiplying the A_{260} by the dilution factor and the extinction coefficient. (1 A_{260} = 40 µg RNA/mL)

A_{260} X dilution factor X 40 = µg 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.

2. Total RNA yield

Total RNA yield will widely vary according to the type and amount of sample. Estimates of expected yield follow:

- ~1 µg RNA per 10^5 eukaryotic cells in culture
- 1–10 µg RNA per mg tissue

Researchers accustomed to working with tissues such as liver or kidney where RNA is plentiful may have unrealistically high expectations of RNA yields from tissues such as muscle, lung, or brain.

3. RNA quality and downstream applications

RNA purity

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

Overall RNA quality

Gel Electrophoresis: The overall quality of a total RNA preparation can be assessed by electrophoresis on a denaturing formaldehyde or glyoxal-based agarose gel (see Figure 2A). High quality total RNA will have fairly compact rRNA bands, and the 28S rRNA band will be about twice as intense as the 18S rRNA band. RNA degradation will cause smearing of the rRNA bands. Glyoxal-based gels eliminate the safety

issues associated with formaldehyde-based gel systems, and they may produce sharper bands. Either type of denaturing agarose gel analysis will also give some information about RNA yield and DNA contamination. Yield of total RNA can be estimated by comparing the staining of rRNA with a sample of known concentration or with a molecular weight marker. DNA, if present, will be evident as a high molecular weight smear or band migrating more slowly than the 28S rRNA band.

Bioanalyzer analysis: The integrity of total RNA can also be evaluated by capillary electrophoresis using an Agilent 2100 bioanalyzer. A 28S:18S rRNA peak ratio at or near 2:1 indicates that the RNA is intact.

Gel analysis of small RNAs: To visualize small RNAs use a denaturing 15% polyacrylamide gel. A procedure is provided in section V.B on page 24. As shown in Figures 2 and 3 on page 5, high quality small RNA samples typically show two sharp bands representing 5S and 5.8S rRNAs, and a more diffuse tRNA band.

RT-PCR and trace genomic DNA contamination

RNA isolated with the *mir*Vana PARIS Kit can be used for most common applications without further processing. DNase I treatment, however, is recommended for RNA that will be used for RT-PCR. It is especially important when using primers that do not flank introns, or for genes that have processed pseudogenes, because the RT-PCR products from RNA and contaminating DNA cannot be distinguished by size in these cases. The DNase I and divalent cations introduced by the DNase I Buffer must be removed from the RNA sample prior to RT-PCR. Divalent cations could degrade RNA at temperatures typically used for RNA denaturation prior to reverse transcription and residual DNase I could degrade DNA made in the RT-PCR. Ambion® TURBO DNA-*free*™ and DNA-*free*™ DNase Treatment and Removal Reagents were designed for this application.

B. Evaluating Yield and Quality of Protein Samples

1. Storage of protein samples

Protein samples can be stored at –20°C for up to 1 week but should be snap frozen in liquid nitrogen and stored at or below –70°C for longer storage, or for use in functional assays. Occasionally residual nucleic acids in the sample may form a white precipitate upon thawing. This precipitate can be removed by centrifugation at 4°C for 1–2 min at top speed in a microcentrifuge without affecting the protein in the sample.

2. Protein concentration

The concentration of protein samples can be assessed using any standard protein quantitation procedure, including the BCA method, the Bradford assay, the Lowry assay or other commercially available protein quantitation assays. The protein concentration can be manipulated during the *mir*Vana PARIS procedure if desired, by varying the volume of Cell Disruption Buffer used for cell or tissue disruption.

For analyses such as gel shift assays or enzymatic assays, protein is usually concentrated enough to be diluted in the reaction buffer. If necessary, however, samples can be concentrated using standard procedures such as acetone or TCA precipitation, vacuum drying, or centrifugal concentrators.

3. Protein yield

The yield of protein is dependent on the tissue or cell line used in the procedure. For eukaryotic cultured cells, in general expect ~200–400 µg protein or more per 10^6 cells.

4. Protein quality and downstream applications

Total protein samples can be used directly for applications such as immunoprecipitation and Western blotting that can tolerate the salt and detergent carried over from the *mir*Vana PARIS procedure. For use in applications such as enzymatic assays, gel shift assays, and two-dimensional gel electrophoresis, it may be necessary to further purify protein samples.

Removing reagents carried over from the *mir*Vana PARIS procedure

If salts and/or detergents present in the Cell Disruption Buffer interfere with your experiments, they can be removed by dialysis or by protein purification on gel-filtration columns.

For 2D gel electrophoresis, treat samples with DNase

Protein samples from whole cells and from nuclear cell fractions contain all of the cellular genomic DNA and can be very viscous. For two-dimensional gel electrophoresis analysis, we recommend treating the samples with DNase 1. Ambion TURBO DNase, which is extremely tolerant of salt and detergent, can be added directly to the Cell Disruption Buffer to remove genomic DNA. Alternatively, protein samples can be treated to remove salts and detergents so that they can be digested with ordinary DNase I.

IV. Troubleshooting

A. Poor RNA Quality

Please see our website for comprehensive information on maximizing RNA yield and quality. Go to www.invitrogen.com/ambion, then type "disruption" into the search engine.

1. RNA looks degraded on a gel

a. Ribosomal RNA is overloaded

Running more than about 5 µg of RNA in a single lane may cause smiling and/or smearing of the rRNA bands. Remember, rRNA comprises about 80% of total RNA, so loading 5 µg of total RNA in a single gel lane will give almost 2 µg of nucleic acid in each major rRNA band.

b. Samples are incompletely denatured

Most RNA forms extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to size. To completely denature RNA, the sample must be both adequately diluted in the gel loading dye, and incubated for enough time at an elevated temperature.

- with Ambion® NorthernMax® Formaldehyde Load Dye (P/N AM8552) for denaturing formaldehyde agarose gels: mix at least 3 volumes Formaldehyde Load Dye with the RNA, and then incubate at 65°C for ~15 min.
- with Ambion NorthernMax® Glyoxal Load Dye (P/N AM8551) denaturing load dye for glyoxal agarose gels: use equal volumes RNA and Glyoxal Load Dye, and incubate 30 min at 50°C.
- with Ambion Gel Loading Buffer II (P/N AM8546G or AM8547) for denaturing polyacrylamide gels: mix a least one volume Gel Loading Buffer II with the RNA, and then incubate for 2–5 min at 95–100°C.

Also, be sure to include a positive control RNA on the gel so that unusual results can be attributed to a problem with the gel or a problem with the RNA being analyzed. RNA molecular weight markers, an RNA sample known to be intact, or both, can be used for this purpose.

c. Agarose gel was run too fast

Smearing may occur if denaturing agarose gels are run at more than 5–6 volts/cm as measured between the electrodes. For example, if the distance between the electrode wires in the electrophoresis chamber measures 15 cm, the gel should be run at a constant 75 volts. For long runs (>3 hr) the buffer may need to be circulated to avoid the formation of pH gradients in the gel.

d. Acrylamide gel troubleshooting

It is important to rinse the wells of denaturing acrylamide gels thoroughly before loading samples to avoid band smearing. Also, to maximize resolution of bands on denaturing acrylamide gels, pre-run the gel for at least 30 min to bring the gel to the running temperature.

2. RNA is degraded

a. Improper handling of sample

It is extremely important to collect, process, and homogenize samples as quickly as possible to avoid RNA degradation by endogenous ribonucleases. It is also very important to keep the samples and the Cell Disruption Buffer as cold as possible (i.e. on ice). If desired, RNase inhibitors such as SUPERase•In can be added at 100–1000 U/mL to the Cell Disruption Buffer just before use.

Immediately after homogenization, lysate should be mixed with the 2X Denaturing Solution at room temperature. This solution contains guanidinium thiocyanate, a strong chaotropic denaturant which rapidly inactivates RNases.

b. Exogenous RNase contamination

Once the lysate is bound to the filter in the Filter Cartridge, and the Denaturing Solution has been washed away, all the typical precautions against RNase contamination should be rigorously observed. Wear gloves at all times, and change them frequently to avoid introducing "finger RNases". Keep the bags containing the Collection Tubes, and the solution bottles closed when they are not in use to avoid contamination with dust. Any tubes or solutions not supplied with the kit, which will contact the RNA, should be bought or prepared so that they are free from RNases.

B. Poor Protein Quality

1. No protein activity or poor protein activity

a. Improper handling of sample

It is important to keep samples cold during the procedure. Keep samples on ice between each step and centrifuge at 4°C when required. Also, complete the procedure as quickly as possible to get the highest quality protein (and RNA).

To store protein samples, snap-freeze them in liquid nitrogen and store below –70°C for use in functional assays.

b. Protein degradation or modification

If required, protease inhibitors, phosphatase inhibitors, and anti-oxidants can be added to the Cell Disruption Buffer just before use. These reagents will not adversely affect subsequent RNA isolation from samples.

2. Low protein concentration

a. Incomplete sample disruption

Incomplete sample homogenization will result in only partial recovery of protein. Some tissues may be more difficult to disrupt than others, especially if they are frozen. Increase the duration of mechanical homogenization in step II.D.2 on page 11, and the subsequent incubation on ice in step II.D.3.b on page 12 if required.

If including a centrifugation step to clarify the protein lysate, be sure that the sample is thoroughly homogenized before centrifugation.

b. Volume of Cell Disruption Buffer was too high

As little as 100–300 µL of Cell Disruption Buffer can be used in the *mir*Vana PARIS procedure to maximize protein concentration. For most cell types, we recommend using at least 300 µL of buffer when isolating RNA and protein from $\geq 10^6$ cells. The buffer volume should be sufficient to resuspend the cells by gentle vortexing.

On the other hand, if too little Cell Disruption Buffer is used, the sample lysate may be too viscous to process properly, thus compromising RNA yield and quality.

V. Additional Procedures

A. Isolation of Small RNAs from Total RNA Samples

The enrichment procedure for small RNAs described in section II.F.II starting on page 15 can be used to enrich total RNA samples for the small RNA fraction (<200 nt). The total RNA sample is simply mixed with 2X Denaturing Solution and sequentially bound to two filters without phenol extraction. The end product is two fractions enriched either in large (first filter) or small (second filter) RNA species that can be used in independent experiments.

1. Mix 100–625 µL of total RNA sample with an equal volume of 2X Denaturing Solution.
   If the RNA sample volume is <100 µL, add Cell Disruption Buffer to bring the sample to 100 µL, then add 100 µL 2X Denaturing Solution.
2. Add 1/3 volume 100% ethanol to the mixture, and mix thoroughly. For example if a 100 µL RNA sample was mixed with 100 µL of 2X Denaturing Solution, then add 67 µL 100% ethanol. Mix thoroughly by vortexing or inverting the tube several times.
3. Continue the procedure starting at step II.F.2 on page 16.

B. Gel Analysis of Small RNAs

The best way to quickly analyze small RNAs is to run an aliquot of the prep on a denaturing 15% polyacrylamide gel (see section V.D on page 26 for recipes).

1. Pour a denaturing 15% polyacrylamide gel, and for maximum resolution of bands, let it polymerize for an hour before assembling it into the electrophoresis device. We also recommend prerunning the gel for 30 min to 1 hr to equilibrate it to the running temperature. If necessary because of time constraints, the gel can be loaded and run immediately after it solidifies.
2. Mix 0.5–2 µg of the RNA with an equal volume of denaturing gel loading buffer. We offer Gel Loading Buffer II (P/N AM8546G or AM8547).
3. Heat sample for 2–5 min at 95–100°C.
4. Rinse the wells thoroughly with 1X TBE, then load the samples and electrophorese at 30–45 mA in 1X TBE.
5. Stop electrophoresis when the bromophenol blue dye front has migrated to the bottom of the gel.
6. Soak the gel for 5 min in a 0.5–1 µg/mL solution of ethidium bromide in 1X TBE.

7. Rinse the gel for 2–5 min in 1X TBE.
8. Visualize the RNA using a UV transilluminator. High quality small RNA will have clearly visible tRNA, 5S rRNA, and 5.8S rRNA bands (see Figures 2 and 3 on page 5).

C. Northern Blot Analysis of Small RNA Molecules

Northern blots can provide qualitative and quantitative data for relatively abundant small RNAs using 1–50 µg of total RNA. For less abundant RNAs, such as specific miRNAs, we recommend using the more sensitive *mir*Vana miRNA Detection Kit with 0.1–5 µg of total RNA. Of course the sensitivity of both blot and solution hybridization analysis of small RNAs can be increased by analyzing RNA fractions that are enriched in small RNA species instead of total RNA.

Specific small RNAs can be detected on Northern blots using either antisense RNA probes prepared by in vitro transcription (*mir*Vana miRNA Probe Construction Kit) or 5' end labeled antisense RNA or DNA probes (*mir*Vana Probe & Marker Kit). Below we provide a procedure using the Ambion® optimized NorthernMax® reagents for RNA probes, and a procedure adapted from Patterson and Guthrie for DNA probes (Patterson 1987).

To look for larger RNAs (e.g. mRNAs which are not resolved on an acrylamide gel) in the same RNA samples, you would have to prepare a Northern blot using an ***agarose*** gel system (Ambion NorthernMax Kits P/N AM1940, AM1946 are ideal for this).

1. Run RNA sample on an acrylamide gel

Run 1–50 µg of total RNA or an RNA fraction enriched in small RNAs on a denaturing 15% polyacrylamide gel (see section V.D for recipes). Stop electrophoresis when the bromophenol blue dye-front has migrated to the bottom of the gel. Stain the gel with ethidium bromide, and examine it on a transilluminator to assess the quality of the samples and to make sure that there is good separation of the RNA.

2. Transfer RNA to hybridization membrane

After staining, transfer the RNA to a nylon membrane by electroblotting (We offer BrightStar®-Plus Nylon membrane P/N AM10100). We typically electroblot in a semi-dry apparatus using a stack of three sheets of blotting paper soaked in 0.25X TBE placed above and below the gel/membrane. For 0.75 mm gels, transfer at 200 mA for at least 0.2 A-hr (A x hr). Extending this time does not result in loss of sample. After blotting, keep the membrane damp and UV crosslink the RNA to the membranes using a commercial crosslinking device (120 mJ burst over 30 sec).

3a. RNA probes

a. Prehybridize membrane in ULTRAhyb-Oligo Hybridization Buffer (P/N AM8663) for at least 1 hr at 65°C.

b. Add 1–5 x 10^5 cpm RNA probe per mL of ULTRAhyb-Oligo.

c. Hybridize 8–24 hr at 42°C.

d. Wash 3 times for 5 min each at room temp with NorthernMax Low Stringency Wash Buffer (P/N AM8673).

e. Wash 15 min at 42°C in NorthernMax Low Stringency Wash Buffer.

3b. DNA probes

a. Prehybridize membrane in prehybridization solution (recipe in section V.D) for at least 1 hr at 65°C.

b. Discard the prehybridization solution.

c. Add 1–5 x 10^5 cpm DNA probe per mL of hybridization solution (recipe in section V.D).

d. Hybridize 8–24 hr at room temp.

e. Wash 3 times for 5 min each at room temp with Northern wash solution (recipe in section V.D).

f. Wash 15 min at 42°C with Northern wash solution.

4. Expose the membrane

After the final wash, wrap the blot in plastic wrap and expose to X-ray film or a phosphorimager screen according to the manufacturer instructions. Phosphorimaging allows quantitation of the amount of signal present in each band.

D. Additional Recipes

1. Recommended polyacrylamide concentration

Nucleic Acid Size	Polyacrylamide Concentration	Xylene Cyanol Position	Bromophenol Blue Position
>30 nt	12%	~40 nt	~15 nt
<30 nt	15%	~30 nt	~10 nt

2. 10X TBE

TBE is generally used at 1X final concentration for preparing gels and/or for gel running buffer.

IMPORTANT

Do not treat TBE with diethylpyrocarbonate (DEPC).

Concentration	Component	for 1 L
0.9 M	Tris base	109 g
0.9 M	Boric Acid	55 g
20 mM	0.5 M EDTA solution	40 mL

Dissolve with stirring in about 850 mL nuclease-free water. Adjust the final volume to 1 L.

Alternatively, we offer nuclease-free solutions of 10X TBE (P/N AM9863, AM9865) and ready-to-resuspend powdered 10X TBE packets (P/N AM9864). Both are made from of ultrapure molecular biology grade reagents.

3. Denaturing acrylamide gel mixes

Use the following instructions to prepare 15 mL of gel mix with the indicated percentage of acrylamide and 8 M urea. 15 mL is enough gel solution for one 13 x 15 cm x 0.75 mm gel. See our product catalog at www.invitrogen.com to find reagents for acrylamide gel preparation.

Amount		
12% gel	15% gel	Component
7.2 g	7.2 g	Urea
1.5 mL	1.5 mL	10X TBE
4.5 mL	5.6 mL	40% acrylamide (acrylamide:bis acrylamide = 19:1)
to 15 mL	to 15 mL	Nuclease-free water
Stir to mix, then add:		
75 µL	75 µL	10% ammonium persulfate
15 µL	15 µL	TEMED

Mix briefly after adding the last 2 ingredients, and pour gel immediately.

4. Denaturing gel loading buffer for polyacrylamide gels

Amount	Component
95 %	deionized formamide
18 mM	EDTA
0.025 %	xylene cyanol
0.025 %	bromophenol blue
0.025 %	SDS

Alternatively, we offer ready-to-use Gel Loading Buffer II (P/N AM8546G or AM8547).

5. 50X Denhardt's Solution

Amount	Component
10 g	Ficoll 400
10 g	bovine serum albumin
10 g	polyvinylpyrrolidone
to 1 L	nuclease-free water

6. 20X SSC

Amount	Component
175.3 g	NaCl
88.2 g	sodium citrate
800 mL	nuclease-free water
pH to 7.0 with HCl	
to 1 L	nuclease-free water

Alternatively, we offer ready-to-use 20X SSC (P/N AM9763, AM9770) or ready-to-resuspend powder (P/N AM9764).

7. Northern blot solutions for DNA probes

Prehybridization Solution
6X SSC
10X Denhardt's Solution
0.2% SDS

Hybridization Solution	
6X SSC	Add 1–5 x 10^6 cpm/mL 5' end labeled antisense DNA probe. Filter solution with 0.45 µm pore filter before use to remove background flecks.
5X Denhardt's Solution	
0.2% SDS	

Wash Solution
6X SSC
0.2% SDS

VI. Appendix

A. References

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-Van Dillen PME, and Van Der Noordaa J (1990) Rapid and Simple Method for Purification of Nucleic Acids. *Journal of Clinical Microbiology.* **28.3**: 495–503.

Chomczynski P and Sacchi N (1987). Single-step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Analytical Biochem.* **162**: 156–159.

Patterson B and Guthrie C (1987). An essential yeast snRNA with a U5-like domain is required for splicing in vivo. *Cell.* **49(5)**:613–24.

B. Quality Control

Functional Testing

Following the procedure in section II, a yield of >10 µg total RNA and >200 µg total protein is obtained per 10^6 HeLa cells. RNA quality and recovery of small RNA is checked by denaturing gel electrophoresis. Protein integrity is checked by Western blot analysis

Nuclease testing

Relevant kit components are tested in the following nuclease assays:

RNase activity
Meets or exceeds specification when a sample is incubated with labeled RNA and analyzed by PAGE.

Nonspecific endonuclease activity
Meets or exceeds specification when a sample is incubated with supercoiled plasmid DNA and analyzed by agarose gel electrophoresis.

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

Protease testing

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

C. Safety Information

WARNING

GENERAL SAFETY. *Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.*

- *Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.*
- *Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.*

1. Chemical safety

WARNING

GENERAL CHEMICAL HANDLING. *To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:*

- *Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.*
- *Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).*
- *Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).*
- *Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.*
- *Handle chemical wastes in a fume hood.*
- *Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)*
- *After emptying a waste container, seal it with the cap provided.*
- *Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.*
- *Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.*
- ***IMPORTANT!*** *Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.*

2. Biological hazard safety

WARNING

Potential Biohazard. *Depending on the samples used on the instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.*

WARNING

BIOHAZARD. *Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:*

In the U.S.:

- *U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: www.cdc.gov/biosafety*
- *Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html*
- *Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.*
- *Additional information about biohazard guidelines is available at: www.cdc.gov*

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

VII. Documentation and Support

A. Obtaining SDSs

Safety Data Sheets (SDSs) are available from:

www.invitrogen.com/sds

or

www.appliedbiosystems.com/sds

Note: For the SDSs of chemicals not distributed by Life Technologies, **contact the chemical manufacturer.**

B. Obtaining support

For the latest services and support information for all locations, go to:

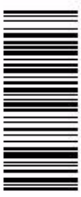
www.invitrogen.com

or

www.appliedbiosystems.com

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches



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