Micro RNA Isolation Kit

INSTRUCTION MANUAL

Catalog #200344

Revision A

BN# 200344-13

For In Vitro Use Only

200344-13

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Micro RNA Isolation Kit

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Micro RNA Isolation Kit

MATERIALS PROVIDED

Material provided[a]	Quantity
Denaturing solution[b]	50 ml
2 M Sodium acetate (pH 4.0)	5 ml
Chloroform:isoamyl alcohol	10 ml
Isopropanol	50 ml
Phenol (equilibrated to pH 5.3–5.7 with 0.1 M succinic acid)	50 ml
β-Mercaptoethanol (β-ME) (14.4 M)	360 µl
Glycogen	50 µl (2 mg/ml)

[a] Sufficient reagents are provided to isolate total RNA from 5 grams of tissue. This kit may be used for one hundred 50-mg (sample weight) isolations or five hundred 10-mg reactions. The choice of isolation size depends on the amount and type of biological sample available and the eventual uses of the RNA.

[b] The composition of this solution can be found in *Preparation of Reagents*.

STORAGE CONDITIONS

Glycogen: –20°C
β-Mercaptoethanol (•-ME): 4°C
Phenol Equilibrated with Succinic Acid: 4°C
All Other Components: Room Temperature

Caution *Chloroform:isoamyl alcohol is flammable and toxic. Phenol is a toxic corrosive. Guanidine isothiocyanate is an irritant. Formaldehyde is a flammable carcinogen.*

ADDITIONAL MATERIALS REQUIRED

Diethylpyrocarbonate (DEPC)
DEPC-treated water
75% (v/v) ethanol and 25% (v/v) DEPC-treated water solution
Liquid nitrogen (for tissue samples only)
Dounce, micro-Dounce, or mechanical microhomogenizer (for tissue samples only)
1× Phosphate-buffered saline (PBS)[§] (for collecting adherent cells only)
1× Trypsin and ethylenediaminetetraacetic acid (EDTA) solution[§] (for collecting adherent cells only)

[§] See *Preparation of Reagents*.

Revision A
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INTRODUCTION

Purification of undegraded RNA from biological sources is central to the investigation of gene expression and regulation. The quality of cDNA libraries, cDNA/polymerase chain reaction (PCR) amplifications, and Northern blot hybridizations depends on the quality of the isolated RNA.

Ribonucleases are found in virtually all tissues and represent the major difficulty in RNA isolation. Guanidine isothiocyanate is one of the strongest protein denaturants and has been used by Chirgwin *et al*. to isolate undegraded RNA from tissue rich in ribonucleases.[1]

The Micro RNA Isolation Kit[2] utilizes a powerful guanidine isothiocyanate–phenol:chloroform extraction,[3] which allows the rapid isolation of RNA. The conditions for extraction enable the partitioning of proteins and DNA into the organic layer of the biphasic solution interface, while retaining RNA in the upper aqueous layer. The aqueous phase is removed to a second tube, and RNA is precipitated with an equal volume of isopropanol.[3] High yields of pure, undegraded total RNA can be recovered from even small quantities of tissue or cells. Large numbers of samples may be performed simultaneously, because of the simplicity of the technique.

The Micro RNA Isolation Kit is a perfect system for extracting RNA from very small samples in only 30 minutes. When biological samples are present in small amounts or when experiments require only small amounts of RNA, the kit will generate pure, undegraded RNA that can be used for cDNA synthesis, PCR amplification, and other applications.

PREPROTOCOL CONSIDERATIONS

Important Precautions

Ribonucleases are very stable enzymes responsible for RNA hydrolysis. The structure of RNase A can be temporarily denatured with extreme conditions, but it readily renatures. RNase A can therefore survive autoclaving and other standard methods of protein inactivation. Since RNases are present in the oils of the skin, **gloves should be worn at all times.**

Note *Rinse off any excess powder on gloves before handling materials and equipment.*

Micropipet bores can be a rich source of RNase contamination, since material accidentally drawn into the pipet or produced by gasket abrasion can fall into RNA solutions during resuspension.

Note *Follow the micropipet manufacturer's recommendations for cleaning. We recommend including an ethanol or methanol rinse of both the interior and exterior of the micropipet shaft.*

Use sterile tubes and micropipet tips handled with gloves only. Avoid equipment or areas which have been used with RNases (e.g., microcentrifuge tubes used for DNA preparation which may have contained concentrated RNase mixtures.)

If the samples are to be converted to cDNA for cDNA library construction or PCR amplification, it is important to remove any residual nucleic acid from the homogenizer and tubes which may remain from previous nucleic acid isolations.

Sterilizing Plasticware and Metal

Plasticware

Disposable, sterilized plasticware is usually free of RNases. If unavailable, components such as 1.5-ml microcentrifuge tubes can be sterilized and treated with diethylpyrocarbonate (DEPC), which chemically modifies and inactivates enzymes.

Note *DEPC is toxic and extremely reactive. Always use DEPC in a fume hood.* ***Read and follow the manufacturer's safety instructions.***

1. Add DEPC to a beaker filled with a known volume of deionized water, yielding a final concentration of 0.1% (v/v).

2. Place the plasticware to be treated into a separate autoclavable container. Mix the DEPC solution and carefully pour it into the container until the plasticware is submerged.

3. Leave the container and the beaker in the fume hood overnight.

4. Pour the DEPC–water off of the plasticware and into another container with a lid. Autoclave the bottle of waste DEPC–water and the container with the plasticware for at least 30 minutes. Aluminum foil may be used to cover the container, but it should be handled with gloves and cut from an area which has not been touched by ungloved hands.

Metal

RNase on glassware or metal is inactivated by baking for a minimum of 3 hours at 250°C.

RNA and RNase Levels in Different Cells

The amount of RNA available for isolation varies with cell types. Liver and kidney cells are metabolically active and produce relatively large amounts of RNA per gram of tissue. Structural cells and fat cells may have less RNA per gram of tissue. The amounts of nuclease in different tissue types also varies. The pancreas and spleen are very rich in nucleases and should be processed or flash-frozen in liquid nitrogen as rapidly as possible.

PROTOCOLS

See the back page of this manual for a condensed, quick-reference protocol that can be detached and used when detailed instructions for these procedures are no longer needed.

Micropreparation of RNA from Animal Tissue Samples

Note *If less than 10 mg of tissue is available, see the note below step 10.*

1. To reduce RNA degradation, flash-freeze tissue samples in liquid nitrogen as soon as possible after removal from the organism.

2. Once frozen, quickly weigh the sample and return it to liquid nitrogen or dry ice.

   WARNING! ***Denaturing solution contains the irritant guanidine isothiocyanate.***

TABLE I

Processing Volumes for Animal Tissue

Tissue Amount	10 mg	20 mg	30 mg	40 mg	50 mg
Denaturing solution	100 µl	200 µl	300 µl	400 µl	500 µl
β-ME	0.72 µl	1.44 µl	2.16 µl	2.88 µl	3.6 µl
2M NaOAc	10 µl	20 µl	30 µl	40 µl	50 µl
Phenol (equilibrated with succinic acid)	100 µl	200 µl	300 µl	400 µl	500 µl
Chloroform:isoamyl alcohol	20 µl	40 µl	60 µl	80 µl	100 µl
Isopropanol	100 µl	200 µl	300 µl	400 µl	500 µl
75% wash	200 µl	400 µl	600 µl	800 µl	1000 µl
DEPC-H_2O	50 µl	100 µl	150 µl	200 µl	250 µl

3. Prepare solution D by adding 7.2 µl of β-ME for every ml of room temperature denaturing solution (see Table I for volumes and amounts). Make enough solution D to yield a solution of 0.1 mg/µl when the tissue is homogenized (e.g., 50 mg of tissue requires 500 µl of solution D).

   Note *If frozen promptly, the homogenate can be stored at –80°C for future use. If the sample is greater than 50 mg in weight, use the appropriate amount of solution D to yield a concentration of 0.1 mg/µl. But when processing, only use 500 µl of sample* ***per microcentrifuge tube*** *and follow the 50 mg guidelines in the chart below.*

4. Place the tissue into the tube containing solution D which will be used for homogenization. Homogenize the frozen tissue sample in solution D using a Dounce, micro-Dounce, or a mechanical microhomogenizer.

5. Transfer the homogenate to a microcentrifuge tube.

   Note *Hereafter, the volume of homogenate used is defined as "volume D" (e.g., in the sample described above in step 3, volume D is 500 µl).*

6. Add a volume of 2 M sodium acetate (pH 4) equal to 1/10th of volume D.

7. Add a volume of phenol (equilibrated to pH 5.3–5.7 with succinic acid) equal to volume D.

   Notes *The provided phenol is equilibrated with succinic acid and is contained in the bottom phase of the bottle (under the aqueous phase). Take care to pipet the bottom phase containing the phenol and to avoid the aqueous phase. If the phenol and aqueous phases are not clear and distinct, incubate the phenol at room temperature or heat the phenol at 37°C until two distinct phases are present.*

   The phenol can be stored at room temperature for up to 3 months. For longer periods, store at 4°C.

8. Add a volume of chloroform:isoamyl alcohol equal to 1/5th of volume D. Cap tightly and vortex vigorously.

9. Spin the mixture in a microcentrifuge for 5 minutes at maximum speed (13–14,000 rpm). Two phases should be clearly visible. Some DNA and protein material may be present at the interphase layer.

10. Carefully transfer the upper phase with the RNA, making sure not to take any material from the interphase layer, to a sterile, RNase-free microcentrifuge tube. Discard the lower level containing phenol, proteins and DNA.

    Note *If RNA is being isolated from less than 10 mg, or if the tissue sample was composed mostly of cells containing relatively small amounts of RNA per unit of wet weight (e.g. fat cells), we recommend coprecipitating the samples with glycogen carrier in the amount equal to 1/100 of volume D. Mix the glycogen carrier with the RNA solution and add isopropanol as described below in step 11. Centrifuge the sample for 30 minutes for maximum precipitation efficiency. Most samples can be efficiently processed without these steps.*

11. Add a volume of isopropanol equal to volume D to the RNA solution. Mix by inversion.

12. Spin the samples in a microcentrifuge at maximum speed (13–14,000 rpm) for 5 minutes.

    Note *Up to this point, the RNA has been protected from ribonucleases by the presence of guanidine isothiocyanate. To ensure against ribonuclease contamination, wear gloves when handling the sample.*

13. Remove the supernatant as completely as possible.

14. Wash the pellet with 75% ethanol–25% DEPC-treated water equal to twice the amount of volume D.

15. Remove the wash completely and dry the pellet under vacuum for 5 minutes. Do not overdry the sample, or it will be difficult to resuspend.

    Note *The volumes given below for RNA resuspension are suggestions. If the end use of the product requires very concentrated solutions of RNA, resuspend the RNA in a smaller volume of DEPC-treated H_2O. Expected yields per mg of mouse tissue are liver, 6–7 μg; kidney, 3–4 μg; skeletal muscle, 1–3 μg; and brain 1–1.5 μg.*

16. Resuspend the RNA in DEPC-treated water equal to 1/2 of volume D. If the material is difficult to resuspend, heat the sample at 68°C for 10 minutes with intermittent vortexing. If the RNA solution appears to be too concentrated, add another aliquot of DEPC-treated water equal to 1/2 of volume D. Mix vigorously.

17. Accurate spectrophotometric measurement of RNA with 500-µl quartz cuvettes require at least 1 µg of RNA. To quantify the RNA, remove a small sample and dilute it with 5T**.**1E or a 5 mM Tris-HCl, pH 7.5 solution. Measure the optical density (OD) at 260 nm and 280 nm to quantify and qualify the RNA. See Appendix I: *Spectrophotometric Measurement of RNA*.

Micropreparation of RNA from Blood Samples

The reagent volumes required to isolate RNA from blood are the same as the values recommended for tissue in Table I, *Processing Volumes for Animal Tissue*. If isolating RNA from 50 µl of blood, use the quantities of reagents listed for 50 mg of tissue. If isolating RNA from 40 µl of blood, use the volumes recommended for 40 mg of tissue, etc. As volumes of blood exceed the recommended levels, the 260/280 ratio decreases, indicating protein contamination. The decrease in purity does not seem detrimental to the cDNA conversion and subsequent PCR amplification, unless the blood volume exceeds 200 µl of blood in the 50 mg of tissue-isolation condition.

The relative RNA yields from blood are considerably less than that of tissue. Roughly 0.45 µg of total RNA can be isolated from 50 µl of blood. After phenol extraction, add 1 µl of glycogen and mix thoroughly prior to isopropanol precipitation. This ensures a visible, firm pellet. While the RNA yield seems small, PCR can easily amplify a moderate-to-abundant RNA (after it is converted into cDNA) from half of the RNA isolated from only 10 µl of blood.

Some anticoagulants present in blood collection vials (i.e., heparin) have been reported to interfere with the cDNA/PCR reaction. These protocols were developed using EDTA as an anticoagulant.

RNA Isolation from Cells Grown in Culture

Adherent Cells

Note *Adherent cells can be removed from the plate with the trypsin procedure listed below, or lysed directly on the plate. Efficient collection of the cell lysate from directly lysed cells may be difficult, due to the viscosity of the solution.*

Direct Lysis of Adherent Cells in the Culture Dish

1. Remove media from the cells. Tilt the plate slightly to remove residual media.

2. Add 500 µl of solution D (3.45 µl of β-ME plus 500 µl of denaturing solution) per 75 cm^2 of cell-covered surface area.

   Example *Use 500 µl of solution D for a 75 cm^2 dish and 1.17 ml of solution D for a flask with 175 cm^2 of surface area.*

3. Spread the solution evenly over the surface. Scrape the cell lysate into a small area and transfer no more than 500 µl of the lysate per microcentrifuge tube. Proceed to step 3 of the protocol on the following page, *Lysis of Cells Grown in Suspension.* The remainder of the protocol is the same.

All downstream processing will relate to the volume of solution D per tube, not cell number.

Collection of Adherent Cells through Trypsin Treatment before Lysis

1. Remove media from the cells by washing the cells with 1/3× media volume of 1 × Phosphate-buffered saline[§] (PBS).

2. Add 1× trypsin–EDTA solution[§] to an amount equal to 1/20th the original media volume.

[§] See *Preparation of Reagents*.

3. Rotate the container, making sure all the cells are covered with the trypsin solution.

4. Allow the cells to incubate at room temperature for 3–5 minutes.

5. Sharply rap the container against a surface (or bump the plate gently against the palm of the hand) to jar the cells loose.

   Note *When held to the light the cells should visibly flow in the container as it is rotated. If the cells are not loose after five minutes, remove the trypsin and place the flask at 37°C for 5 minutes, and repeat step 5.*

6. Add 1× PBS equivalent to 1/2 the original media volume to slow the action of the trypsin. Collect the cells by centrifugation at 4000 × *g* for 5 minutes. Remove the supernatant and proceed with lysis (step 2 of the *Micropreparation of RNA from 1 × 10^6 to 5 × 10^6 Cultured Cells* protocol below). Proceed with the following protocol, *Lysis of Cells Grown in Suspension.*

Lysis of Cells Grown in Suspension

Note *If less than 1 × 10^6 cells are used, follow the procedure for 1 × 10^6 cells and precipitate the RNA with 1 µl of glycogen as carrier. Centrifuge the samples at full speed for 30 minutes.*

TABLE II

Processing Volumes for Cultured Cells

Cell Amount	1 × 10^6 cells	2 × 10^6 cells	3 × 10^6 cells	4 × 10^6 cells	5 × 10^6 cells
Denaturing solution	100 µl	200 µl	300 µl	400 µl	500 µl
β-ME	0.72 µl	1.44 µl	2.16 µl	2.88 µl	3.6 µl
2M NaOAc	10 µl	20 µl	30 µl	40 µl	50 µl
Phenol (equilibrated with succinic acid)	100 µl	200 µl	300 µl	400 µl	500 µl
Chloroform:isoamyl alcohol	20 µl	40 µl	60 µl	80 µl	100 µl
Isopropanol	100 µl	200 µl	300 µl	400 µl	500 µl
75% Wash	200 µl	400 µl	600 µl	800 µl	1000 µl
DEPC–H_2O	25 µl	50 µl	75 µl	100 µl	125 µl

1. Centrifuge the cells at 1000 × *g* for 5 minutes. Remove most of the supernatant. Resuspend the cells in the residual supernatant and transfer them to a microcentrifuge tube. Collect the cells into a loose pellet by briefly pulsing the tubes in a microcentrifuge. Remove all residual supernatant. To prevent a viscous lysis pellet from forming at the bottom of the tube, flick the tube gently to distribute the cells evenly on the walls of the tube.

Note *Although lysis volumes are given for a specific number of cells, individual cell mass can vary significantly. Generally, fibroblasts and carcinoma cell lines have a greater cell mass than cells which grow in suspension. When there is too much cell mass in volume D, the increase in viscosity causes a decrease in RNA yield and an increase in DNA contamination. The volumes in this protocol are given for cells grown in suspension. For larger cells, process 1 × 10^6 cells per tube, using the volumes of reagent recommended for 5 × 10^6 cells.*

2. 100 µl of solution D should be used for every 10^6 cells processed (See Table II above for volumes and amounts). Prepare solution D by adding 7.2 µl of β-ME to every ml of denaturing solution required. The volume of solution D used is referred to as "volume D."

3. Add a volume of 2 M sodium acetate (pH 4.0) equal to 1/10th of volume D.

4. Add a volume of phenol (equilibrated to pH 5.3–5.7 with succinic acid) equal to volume D.

   Note *Before proceeding to step 5 below, note that the phenol is equilibrated with succinic acid. Pipet only the bottom phase containing the phenol.*

5. Add a volume of chloroform:isoamyl alcohol equal to 1/5th of volume D. Cap tightly and vortex vigorously.

6. Spin the mixture in a microcentrifuge for 5 minutes at maximum speed (13–14,000 rpm). Two phases should be clearly visible. Some DNA and protein material may be present at the interphase layer.

7. Carefully transfer the upper phase with the RNA, making sure not to take any of the interphase layer, to a sterile, RNase-free microcentrifuge tube. Discard the lower level containing phenol, proteins and DNA.

   Note *If less than 1 × 10^6 cells were processed, we recommend coprecipitating the samples with glycogen carrier in the amount equal to 1/100 of volume D. Mix the glycogen carrier with the RNA solution and add isopropanol as described in step 8 below. Spin the mixture in a microcentrifuge for 30 minutes for maximum precipitation efficiency. Most samples can be efficiently processed without these additional steps.*

8. Add a volume of isopropanol equal to volume D to the RNA solution. Mix well.

9. Spin the samples in a microcentrifuge at maximum speed (13–14,000 rpm) for 5 minutes. (For samples containing less than 1×10^6 cells, spin the samples in a microcentrifuge for 30 minutes for efficient recovery.)

   Note *Up to this point, the RNA has been protected from ribonucleases by the presence of guanidine isothiocyanate. To ensure against ribonuclease contamination, wear gloves when handling the sample.*

10. Remove the supernatant as completely as possible.

11. Wash the pellet with 75% ethanol–25% DEPC-treated water equal to twice the amount of volume D.

12. Remove the wash completely and dry the pellet under vacuum for 5 minutes. Do not overdry the sample, or it will be extremely difficult to resuspend.

    Note *The volumes given below for RNA resuspension are suggestions. If the end use of the product requires very concentrated solutions of RNA, resuspend the RNA in a smaller volume of DEPC-treated H_2O. Yields for RNA isolations from 10^6 B-cells (Burkitt's lymphoma) are 15–20 μg; from T-cells (human), 12–14 μg; and from murine fibroblasts 5–9 μg.*

13. Resuspend the RNA in DEPC-treated water equal to 1/2 of volume D. If the material is difficult to resuspend, heat the sample at 68°C for 10 minutes with intermittent vortexing. If the RNA solution appears to be too concentrated, add another aliquot of DEPC-treated water equal to 1/2 of volume D. Mix vigorously.

14. Dilute the RNA sample and measure the OD at 260 and 280 nm to quantify and qualify the sample. See Appendix I: *Spectrophotometric Measurement of RNA*.

15. Store the RNA at –20°C or –80°C.

APPENDIX I: SPECTROPHOTOMETRIC QUANTIFICATION OF RNA

Note *Accurate spectrophotometric measurement of RNA in a 500-µl cuvette requires at least 1 µg of RNA. Measurements which register less than optical density 0.05 (OD_{260}) are unacceptable.*

1. Zero the spectrophotometer at 260 nm with DEPC-treated water or 5 mM Tris-HCl (pH 7.5), or TE buffer.

   Note *If the DEPC-treated water has a pH <7, the quantitation should be performed in 5 mM Tris-HCl (pH 7.5), or 5T.1E. The low pH will alter the OD measurements between 260 and 280 nm, indicating a low purity.*

2. If using a 500-µl cuvette, place 5 µl of the RNA solution into 495 µl of the diluent. Place a piece of Parafilm® over the top of the cuvette and mix the sample well. The conversion factor for RNA is 0.040 µg/µl per OD_{260} unit. Take the spectrophotometric reading. For a reading of 0.10, calculate the concentration as follows:

0.10	×	500/5	×	0.04 µg/µl	=	0.4 µg/µl
spec. reading A_{260}		dilution factor		conversion factor A_{260}		final concentration

3. Calculate the yield of RNA by multiplying the volume in microliters by the concentration. For example, in the sample above a volume of 100 µl results in a yield of 40 µg.

4. Rezero the spectrophotometer with desired solution at 280 nm. Calculate the purity of the RNA by measuring the OD at 280 nm. The ratio of the 260 nm measurement to the 280 nm measurement indicates purity. Ratios of 1.8 to 2.0 are very pure. Lower ratios indicate possible protein contamination, or low pH in the solution used as a diluent for the spectrophotometric readings.

APPENDIX II: FORMALDEHYDE GEL PROTOCOL

Preprotocol Considerations

Caution *Formaldehyde is a suspected carcinogen and must be used and disposed of in accordance with federal, state and local regulations. Always use formaldehyde in a fume hood.*

The secondary structure of mRNA present in the total RNA must be denatured if the molecules are to migrate at their true molecular weight. The percentage of agarose used affects resolution and transfer. High agarose concentrations improve resolution but decrease the rate and efficiency of RNA transfer to membranes. For large transcripts (>3500 bases), agarose concentrations should not exceed 0.8%. The following protocol is recommended for most RNA sizes.

Protocol

1. Lyophilize the RNA samples without heat until they are dry. 5–15 µg of RNA works well for most applications. More than 15 µg of RNA may cause the lanes to become distorted with ribosomal RNA.

   Note *The RNA can be dried completely without severe resuspension problems, since the loading buffer contains 48% formamide.*

2. Melt 1 g of agarose in 85 ml of deionized water in a flask.

3. Add 10 ml of 10× MOPS buffer (see *Preparation of Reagents*) to the agarose solution. Allow the gel solution in the flask to cool to 55°C while preparing an electrophoresis gel mold. Place the gel mold on a level space inside a fume hood. Add 5.4 ml of 37% formaldehyde to the cooled agarose. Swirl to mix and quickly pour the agarose into the gel mold. If you wish to transfer the RNA on the gel to a membrane, the gel should only be thick enough to handle easily (0.5–0.75 cm). Insert comb, and allow the gel to solidify in the fume hood.

4. While the gel is solidifying, start a boiling water bath and prepare 5 µl of sample loading buffer for each sample. Prepare the loading buffer by mixing the components listed below (no more than 12 hours before use):

   For 100 µl total volume of loading buffer use:

   48 µl of deionized formamide
   17.3 µl 37% formaldehyde solution
   34.7 µl loading dye (see *Preparation of Reagents*)

   Note *This solution is not stable.* ***Do not*** *use after 12 hours.*

5. Cover the solidified gel with 1× MOPS buffer. Carefully pull the comb out and connect the electrophoresis apparatus to a power supply.

6. Resuspend the lyophilized RNA in 5 µl of loading buffer. Boil for 2 minutes, centrifuge the sample to collect condensation and immediately load onto the gel.

7. Electrophorese the gel at 100 V. Ethidium bromide in the loading dye will migrate to the negative electrode, and the bromphenol blue will travel to the positive electrode with the RNA sample. Run the bromphenol blue one-half to three-quarters the length of the gel (depending on the resolution desired).

   Note *Formaldehyde gels are more fragile than other agarose gels. Use caution when moving the gel. Wear UV-protective safety glasses or a full safety mask to prevent UV damage to the face and skin.*

8. Examine the gel with UV illumination.

Expected Results

The majority of eukaryotic mRNA falls within the size range of 400–2000 bases. If a size marker is unavailable, the upper and lower ribosomal RNA bands can be used to help size the RNA. The large 28s band is ~5 kb, and the smaller 18s band is ~2 kb. These numbers are only approximate, since ribosomal RNA sizes vary between species.

If proceeding with northern blotting, the gel should be photographed alongside a ruler, its zero point placed at the wells. Do not allow the surface of the gel to become dry prior to transfer.

APPENDIX III: NORTHERN TRANSFER PROTOCOL

Preprotocol Considerations

We recommend using systems which transfer RNA before the gel matrix collapses. The protocols in this manual for formaldehyde gels and northern transfer work well, because RNA is loaded in a small volume which immediately sinks to the well bottom. As RNA migrates, it stays in the bottom of the gel. When the gel is blotted, as described below, the gel is placed bottom side up, which requires the RNA to migrate only a short distance before reaching the membrane.

Alternatively, the RNA may be transferred overnight to a solid support through capillary transfer. In this system, a wick continues to supply the gel with buffer as the light pressure of absorbent materials creates a slow capillary flow.

Protocol

1. If the mRNA of interest is >2.5 kb, pretreat the gel by soaking in 0.05 M NaOH for 20–30 minutes. (The alkaline conditions partially hydrolyze the mRNA, improving transfer efficiency.) Follow the alkali step with a 30–minute neutralization in 0.1 M Tris-HCl at pH 7.5, 0.15 M NaCl.

2. Prepare 500 ml of 10× SSC (see *Preparation of Reagents*). Place a solid support, such as a glass plate, over a container on a level surface. Cut a strip of Whatman® 3MM paper long enough to bridge the support and reach the bottom of the container on both sides (see Figure 1, *Capillary Northern Blot Setup*).

3. Wet the Whatman paper with 10× SSC and drape over the support. While wearing gloves, remove any air bubbles trapped between the paper and the glass.

4. Place the gel, well side down, on the Whatman paper. Remove any bubbles trapped between the gel and the Whatman paper.

5. Cover the exposed areas of the Whatman paper that surround the gel with Parafilm or plastic wrap.

   Note *This prevents the absorbent material placed on top of the gel from "short-circuiting" the capillary transfer by coming into contact with wet material. The only connection between the 10× SSC reservoir and the absorbent material should be through the gel.*

6. Cut out a piece of membrane and two pieces of Whatman paper that are the size of the gel or slightly bigger. On the area of the membrane to be placed above the wells, write an identifying label and any information which will assist you in recalling the orientation of the loaded samples.

Note *Nitrocellulose membranes work well for sensitivity but are relatively fragile compared to nylon membranes. If the Northern is to be stripped and reprobed, nylon membrane should be used.*

7. Wet the membrane in water, then in 10× SSC. Place the membrane, writing side down, on top of the gel. Remove any bubbles between the membrane and the gel.

8. Wet the two pieces of Whatman paper in 10× SSC and place them above the membrane. Remove any trapped bubbles.

9. Place 8–10 cm of absorbent material (paper towels or blotting material) on top of the filter paper, placing a weight and a support (not exceeding 800 g in weight) on top of the absorbent material. Check to see that the support is balanced by using a level. This ensures equal blotting over the surface of the gel and prevents the assembly from toppling over.

10. Check the level again after about 30 minutes, because the absorbent material sometimes settles unevenly.

11. Allow the transfer to proceed for 12–18 hours.

12. Remove the absorbent material. Do not remove the two top sheets of Whatman paper. Remove the Parafilm or plastic wrap and cut the filter paper underneath, leaving a border of approximately 2 cm around the gel.

13. Without disturbing any of the intervening components, turn the entire pile, including the bottom sheet of filter paper, upside down. Place on a level surface and gently peel back the filter paper which was on the bottom (now on top).

14 Trace the well locations with a sharp pencil by putting the pencil tip through the wells and marking the membrane (now under the gel).

15. Carefully remove the agarose gel.

16. Check transfer efficiency by staining the gel in ethidium bromide and examine under UV illumination (or shine a hand-held UV lamp directly on the damp membrane).

Caution *Wear UV-protective safety glasses or a full safety mask to prevent UV damage to the face and skin.*

17. The image of the UV-illuminated membrane can now be photographed.

Note *Limit exposure to UV light if the RNA is to be UV-fixed to the membrane, since over-irradiation can cause a decrease in hybridization signal.*

18. Fix the RNA to the membrane permanently by UV-crosslinking or baking under vacuum for 2 hours at 80°C.

19. Store the dry membrane in plastic wrap or a heat-sealable bag until hybridization.

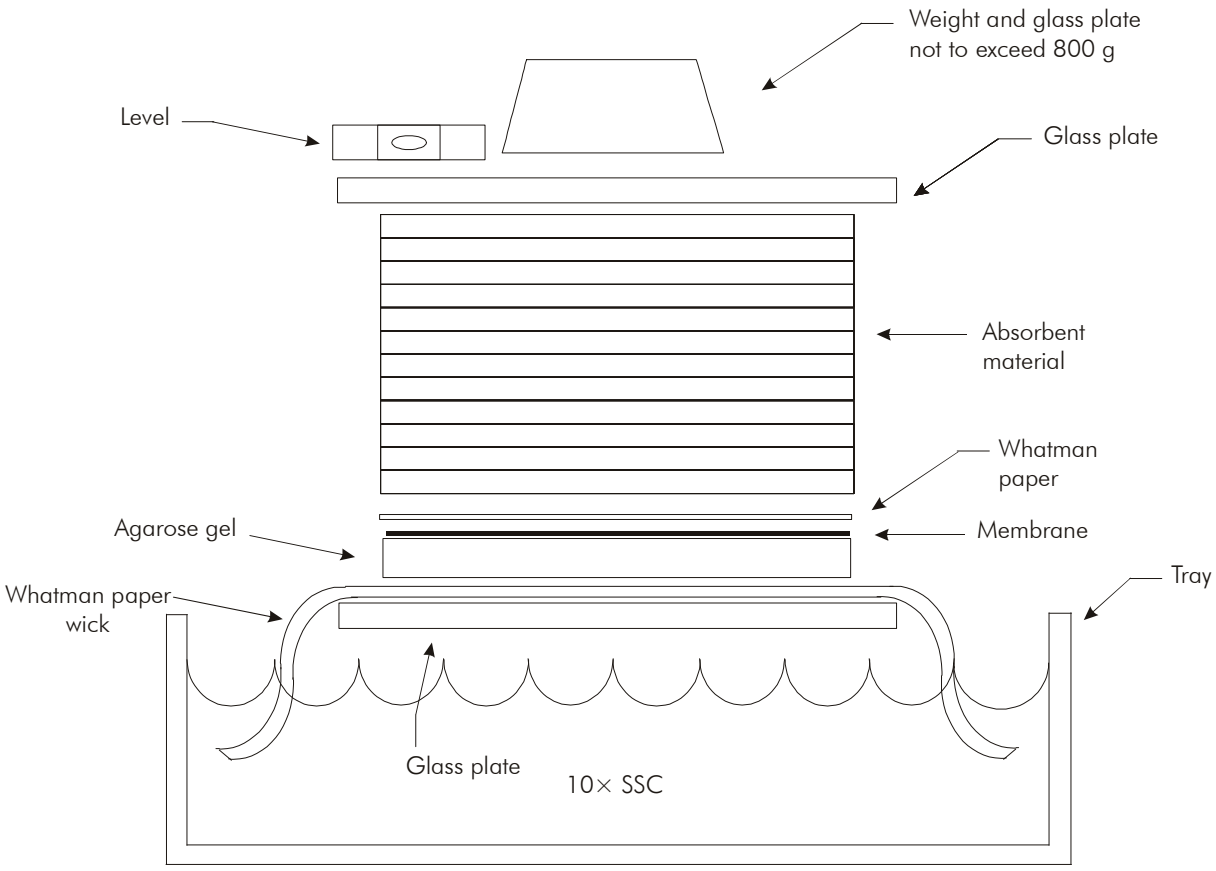

FIGURE 1 Capillary northern blot setup.

APPENDIX IV: HYBRIDIZATION PROTOCOL

Preprotocol Considerations

Greater signal can be detected in less time when the hybridization volume is minimized and the probe has a very high specific activity. Randomly primed probes, such as those produced by the Prime-It II Random Primed Labeling kits (Stratagene Catalog #300385) and hybridization solutions which contain volume excluders, work very well in this application. If using a single-stranded DNA or RNA probe, check to ensure that the probe is complementary, not homologous, to the target mRNA.

Probing with nonradioactive probes can be difficult with northern blots, because the blocking buffers used for the biotin/streptavidin/alkaline phosphatase systems often contain significant amounts of RNase which can degrade the RNA present on the blot. The Stratagene Illuminator nonradioactive detection system (Catalog #300360) is a chemiluminescent probe system for northern blots that can detect RNA messages with sensitivity equivalent to radioactive probes.

Protocol

1. Wet the membrane in deionized water. Gently shake or blot on Whatman paper to remove most of the water.

2. Add the membrane to a container containing an appropriate amount of Stratagene QuikHyb rapid hybridization solution [Catalog #201230 (250 ml) and #201221 (1 liter)] or an appropriate amount of 6× SSC, 2× Denhardt's reagent and 0.1% SDS. Cover the membrane completely with the solution.

3. Prehybridize by shaking or rotating the container with the blot at 68°C for 30 minutes (with QuikHyb solution) or 2 hours (with standard prehybridization solutions).

4. Hybridize at a temperature consistent with probe length and identity. For oligonucleotide probes, the hybridization temperature should be 5–10°C below T_m (see equation below to calculate T_m).

Calculation of the Melting Temperature

OLIGONUCLEOTIDES SHORTER THAN 18 BASES

$$T_m = 2°C(A + T) + 4°C(G + C)$$

OLIGONUCLEOTIDES 14 BASES AND LONGER (UP TO 60–70 NUCLEOTIDES)

$$T_m = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\%\ G + C) - (600/N)$$

where N is the chain length.

For probes with perfect homology longer than 100 bases, hybridization at 68°C works well. For smaller probes or probes with imperfect matches to the target sequence, hybridize at 45°–50°C and use the washing step below (step 8) to remove nonspecific hybridization.

5. Denature the probe by boiling for 5 minutes.

6. Centrifuge the sample to collect condensation, and add the sample to the prehybridizing blot at a concentration of 1–2 × 10^6 cpm/ml of hybridization solution.

7. Hybridize for 1 hour (with QuikHyb solution) or 16–24 hours (with other hybridization solutions).

8. If the probe is short or imperfectly matched to the target, wash 4 times in 2× SSC and 0.1% SDS at 45°C. Otherwise, wash the blot in four 15–minute rinses of 0.1× SSC and 0.1% SDS at 60°C.

9. Expose the blot as described below in step 10. Do not let the blot dry.

   Note *If the blot is to be stripped and reprobed, or washed at higher temperatures, keep the membrane wet. After the membrane dries, the probe can become permanently affixed to the membrane. For best results, seal the wet membrane in a heat-seal bag.*

10. Wrap the membrane in plastic wrap and expose the membrane to X-ray film at –70°C with an intensifying screen for 24–48 hours.

    Note *After the autoradiograph is developed, if significant nonspecific signal is still present, wash the blot at gradually higher temperatures and lower SSC concentrations until the nonspecific signal is gone. Small probes or probes made from degraded template may bind in significant amounts to the ribosomal RNA bands.*

TROUBLESHOOTING

Observation	Suggestion(s)
Interphase layer is not well resolved	β-ME or chloroform:isoamyl alcohol solution was not added to the extraction
	Aqueous layer was not removed immediately after centrifugation. Spin the samples in a microcentrifuge again for 5 minutes
Degraded RNA	RNA is contaminated with RNase. Use DEPC-treated or radiation-sterilized plasticware
	Sample was not processed or frozen fast enough. Some tissues, such as the spleen and pancreas, are very difficult to process without RNase degradation and must be removed from the animal and flash-frozen immediately
	When cultured cells are processed, they should not be removed from the incubator until the last possible moment. Once the media is removed, the cells must be processed rapidly
Poor yields	If too great a mass of cultured cells were processed in a limiting volume, the increased viscosity of the lytic mixture can cause significant loss of RNA into the phenol phase or the interphase layer. Prevent viscosity by processing a less-concentrated sample of cells
	If the mass of cells is unknown, process several cell concentrations. For example: 1×10^6 cells in 100, 300, and 500 µl of solution D. The viscosity of a solution can be decreased by passing the sample several times through an 18–21 gauge needle
DNA contamination	If the lytic solution is too viscous, some DNA may be left in the aqueous layer. To reduce and eliminate viscosity, see the recommendations in the *Poor Yields* Troubleshooting section
	Some of the interphase layer may have been removed with the aqueous layer–see the *Interphase Layer Is Not Well Resolved* Troubleshooting section
	Genomic DNA contamination of RNA isolated with the Micro RNA Isolation Kit is minimal and should not cause a problem with most PCR amplifications. PCR amplification is so powerful that contamination, even with a few molecules of DNA can sometimes be detected. If genomic DNA contamination is a problem with the templates and primers of choice, one of the following strategies might help: 1. Choose primer sets which amplify across splice junctions of processed mRNA, making the unprocessed genomic DNA an unamplifiable target. 2. Choose primer sets which amplify cDNA made from processed mRNA, and not genomic DNA, since the unprocessed template would be too large to PCR-amplify effectively. 3. Choose primer sets to create genomic DNA and the cDNA amplification products of different sizes. 4. DNase-treat the RNA with RNase-free DNase I according to the following protocol: a. Assemble the following reaction in an RNase-free tube: 1–5 µg of total RNA 1 µl of 10× DNase I buffer (see *Preparation of Reagents*) 1 U of DNase I (RNase-free) Bring the volume to 10 µl with DEPC-treated water b. Incubate for 15 minutes at room temperature. c. Add 1 µl of RNase-free 20 mM EDTA to the solution and heat the tube at 65°C for 10 minutes.
PCR amplification of cDNA made from microextracted RNA appears smeared with excessive background bands	Use less RNA as a template for cDNA. When 1 µg of total RNA is converted into cDNA in a 50 µl volume, 1/5 of the cDNA reaction is enough to amplify extremely rare messages.

PREPARATION OF REAGENTS

1× PBS

137 mM NaCl
2.6 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
Adjust the pH to 7.4 with Tris-HCl

1× Trypsin–EDTA Solution

0.05% trypsin
0.53 mM EDTA

10× MOPS Buffer

0.2 M MOPS (3-[N-morpholino] propanesulfonic acid)
0.05 M sodium acetate
0.01 M EDTA
Bring to a final pH of 5.5–7.0 with NaOH.
Do not autoclave

Loading Dye

160 µl 10× MOPS Buffer
100 µl sterile water
100 µl ethidium bromide (10 mg/ml)[||]
80 µl sterile glycerol
80 µl saturated bromphenol blue in sterile water

Note *If the RNA is to be transferred to a solid support (Northern blot), use 5 mg/ml EtBr solution.*

This solution is stable if kept in a light-proof container. Always mix well before using. To make saturated bromphenol blue, add a small amount of bromphenol blue crystals to a microcentrifuge tube and vortex. Centrifuge the sample briefly and look for the presence of a pellet. Do not disturb the pellet when transferring the solution to add to the loading dye.

DNase I Buffer

200 mM Tris-HCl (pH 8.3)
500 mM KCl
25 mM $MgCl_2$
1 mg/ml nuclease-free bovine serum albumin (BSA)

20× SSC Buffer (per Liter)

175.3 g of NaCl
88.2 g of sodium citrate
800.0 ml of water
Adjust to pH 7.0 with a few drops of a 10.0 N NaOH
Adjust volume to 1 liter with water

Denaturing Solution

4 M guanidine isothiocyanate (GITC)
0.02 M sodium citrate
0.5% sarcosyl

REFERENCES

1. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* 18(24):5294-9.
2. Hansen, C. and Braman, J. (1993) *Strategies* 6(2):50-51.
3. Chomczynski, P. and Sacchi, N. (1987) *Anal Biochem* 162(1):156-9.

ENDNOTES

Parafilm® is a registered trademark of the American National Can Company.
Whatman® is a registered trademark of Whatman Paper Ltd.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

An **Agilent Technologies** Division

Micro RNA Isolation Kit

Catalog #200344

QUICK-REFERENCE PROTOCOL

- Collect sample
- Flash-freeze sample in liquid nitrogen; weigh sample
- Prepare enough solution D to process sample

0.72 µl of β–ME for every 100 µl of denaturing solution
100 µl of solution D for every 10 mg or 1×10^6 cells

- Homogenize sample in solution D
- The volume of solution D = Volume D
- Add 1/10 volume D of 2 M sodium acetate
- Add a volume equal to volume D of phenol (equilibrated to pH 5.3–5.7 with succinic acid)
- Add 1/5 volume D of chloroform:isoamyl alcohol;

Cap and vortex thoroughly

- Spin the sample in a microcentrifuge at 13–14k rpm for 5 minutes
- Transfer aqueous layer to a tube containing a volume equal to volume D of isopropanol*
- Spin the sample in a microcentrifuge at 13–14k rpm for 5 minutes
- Remove supernatant completely
- Wash pellet in 2× volume D of 75% ethanol–25% DEPC-treated water
- Remove supernatant completely
- Dry for 5 minutes or less
- Resuspend pellet in a volume equal to 1/2 to 1/10th of volume D in DEPC-treated water

* For samples less than 10 mg or 1×10^6 cells, mix 1 µl of glycogen with the RNA solution before adding the isopropanol. Spin the sample in a microcentrifuge for 30 minutes for maximal recovery of the RNA.