RNA Isolation Kit

Instruction Manual

Catalog #200345

Revision D.0

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RNA Isolation Kit

Contents

RNA Isolation Kit

MATERIALS PROVIDED

Materials Provided[a]	Concentration	Quantity
Denaturing solution	—	100 ml
β-Mercaptoethanol	14.33 M	750 µl
Sodium acetate (pH 4.0)	2 M	7.5 ml
Phenol (pH 5.3–5.7, equilibrated with 0.1 M succinic acid)[b,c]	—	75 ml
Chloroform–isoamyl alcohol	—	15 ml
Isopropanol	—	100 ml

[a] The RNA Isolation Kit provides enough reagents to isolate total RNA from 7 g of tissue or from 7×10^8 tissue culture cells.

[b] The phenol provided with this kit is shipped at room temperature.

[c] Do not use the phenol provided with this kit for DNA isolation.

STORAGE CONDITIONS

β-Mercaptoethanol: 4°C

Phenol (Equilibrated with Succinic Acid): 4°C

All Other Components: Room Temperature

Warning *The denaturing solution contains the irritant guanidine isothiocyanate.*

ADDITIONAL MATERIALS REQUIRED

Tissue grinder with a PTFE (polytetrafluoroethylene) pestle and a glass receptacle **or** a rotating blade homogenizer such as a Polytron® homogenizer
Polypropylene tubes (50 ml)
Ethanol [75% (v/v)] in diethylpyrocarbonate(DEPC)-treated water
DEPC-treated water

Revision D.0

INTRODUCTION

The RNA Isolation Kit minimizes the major difficulty in isolating RNA: the degradation of RNA by ribonucleases (RNases). The kit uses guanidine isothiocyanate[1] (GITC), one of the strongest protein denaturants, to inactivate RNases and protect the RNA from degradation. High yields of high-purity, intact, total RNA are recovered from tissues rich in RNases, from a small amount of tissue, or from a small number of tissue culture cells. The quality of the isolated RNA is sufficient to construct a cDNA library and to isolate a desired clone. The kit uses a single-step, GITC, phenol–chloroform extraction,[2] which allows isolation of RNA in only 4 hours with the standard protocol or in only 60–90 minutes with the alternative time-saving protocol. The method limits handling of the sample and materials, which reduces contamination of samples by RNases and consequent loss and degradation of RNA. Because of the simplicity of the technique and the elimination of ultracentrifugation steps, multiple samples can be run simultaneously.

PROTOCOL GUIDELINES

- Wear gloves at all times during the extraction procedure and while handling materials and equipment to prevent contamination by RNases.
- Exercise care to ensure that all equipment (e.g., the homogenizer, centrifuge tubes, etc.) is as free as possible from contaminating RNases. Treatment of all equipment with diethyl-pyrocarbonate (DEPC)[3] and autoclaving with baking is recommended for all equipment.
- Prepare all solutions with DEPC-treated water, using 0.1% (v/v) DEPC in distilled water (dH_2O). Allow the DEPC-treated water to incubate overnight at room temperature and then autoclave the DEPC-treated water prior to use. If a solution contains Tris base, prepare the solution with DEPC-treated water and autoclave the solution **before** adding Tris base. Autoclave the solution once again after the addition of Tris base to the solution (see *Preparation of Media and Reagents*).

RNA ISOLATION PROTOCOLS

Homogenizing Animal Tissue

This protocol is for isolating total RNA from 1 g of animal tissue.

Note *To reduce RNA degradation, extract RNA from the tissue immediately after dissection from the animal or flash-freeze the tissue sample in liquid nitrogen immediately after dissection.*

1. Prepare solution D by adding 100 µl of β-mercaptoethanol to 14 ml of room temperature denaturing solution. Solution D may be stored at room temperature for up to 1 month.

2. Quickly weigh 1 g of tissue and immediately store the remaining tissue in liquid nitrogen. Immediately place the tissue sample into a tube containing 10 ml of solution D.

3. Mince and homogenize the tissue with 10 ml of solution D in a glass–PTFE homogenizer. Transfer the homogenate to a 50-ml polypropylene tube. Alternatively, homogenize the tissue directly in a 50-ml polypropylene tube with a rotating blade homogenizer such as the Polytron homogenizer or an equivalent instrument. Save the remaining 4 ml of solution D on ice until needed in step 11 of *Isolating RNA*.

4. Proceed with the protocol in *Isolating RNA* or with the *Alternative Time-Saving Protocol for Isolating RNA*.

Preparing Tissue Culture Cells Grown in Suspension

This protocol is for isolating total RNA from 1×10^8 tissue culture cells grown in suspension. Vary the volume of the reagents proportionately to the number of cells in the sample.

1. Prepare solution D by adding 100 µl of β-mercaptoethanol to 14 ml of denaturing solution. Solution D may be stored at room temperature for up to 1 month.

2. Gently pellet the cells by centrifugation and discard the supernatant.

3. Add 10 ml of solution D to the cell suspension and mix thoroughly. Save the remaining 4 ml of solution D on ice until needed once again in step 11 of *Isolating RNA*.

4. Incubate the suspension for 1 minute at room temperature.

5. Transfer the cell suspension to a 50-ml polypropylene tube.

6. Proceed with the protocol in *Isolating RNA* or the *Alternative Time-Saving Protocol for Isolating RNA*.

Preparing Tissue Culture Cells Grown in Monolayer

This protocol is for isolating total RNA from tissue culture cells grown in monolayer and can be used for a maximum of ten 100-mm tissue culture dishes.

1. Prepare solution D by adding 100 µl of β-mercaptoethanol to 14 ml of denaturing solution. Solution D may be stored at room temperature for up to 1 month.

2. Decant the tissue culture medium from the tissue culture plates.

3. Add 10 ml of solution D to a tissue culture plate and swirl the plate gently for 30 seconds. Store the remaining 4 ml of solution D on ice until needed in step 11 of *Isolating RNA*. (For a single tissue culture plate, use 2 ml of solution D and divide the volumes of the reagents used in the remainder of the protocol by 5.)

4. Using a pipet, transfer solution D from the tissue culture plate to a second tissue culture plate and swirl the second plate gently for 30 seconds.

5. Repeat step 4 for the remaining plates.

6. Transfer the cell–solution D mixture in the final plate to a 50-ml polypropylene tube.

7. Proceed with the protocol in *Isolating RNA* or the *Alternative Time-Saving Protocol for Isolating RNA*.

Isolating RNA

1. Add 1.0 ml of 2 M sodium acetate (pH 4.0) to the sample. Mix the contents of the tube thoroughly by repeatedly inverting the tube.

2. Add 10.0 ml of phenol (pH 5.3-5.7) to the tube. Mix the contents thoroughly by inversion.

   Note *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.*

3. Add 2.0 ml of chloroform–isoamyl alcohol mixture to the sample. Cap the tube tightly and shake the tube vigorously for 10 seconds.

4. Incubate the tube on ice for 15 minutes.

5. Transfer the sample to a 50-ml, thick-walled, round-bottom centrifuge tube that has been prechilled on ice.

6. Spin the tube in a centrifuge at 10,000 × *g* for 20 minutes at 4°C.

   Note *After centrifugation, two phases should be clearly visible. The upper, aqueous phase contains the RNA. The lower, phenol phase and interphase contain DNA and proteins.*

7. Taking care to avoid the interphase and the lower phase, transfer the upper, aqueous phase, which contains the RNA, to a fresh centrifuge tube. Discard the lower phenol phase, which contains proteins and DNA.

8. Add an equal volume of isopropanol to the tube that contains the aqueous phase and mix the contents by inversion.

9. Incubate the tube for ≥1 hour at –20°C to precipitate the RNA.

10. Spin the tube in a centrifuge at 10,000 × *g* for 20 minutes at 4°C. After centrifugation, the pellet at the bottom of the tube contains the RNA. Remove and discard the supernatant.

11. Dissolve the pellet in 3.0 ml of solution D. Gently pipet the pellet, if necessary.

12. Add 3.0 ml of isopropanol to the tube and mix the contents well.

13. Incubate the tube for 1 hour at –20°C.

14. Spin the tube in a centrifuge at 10,000 × *g* for 10 minutes at 4°C. Remove and discard the supernatant from the tube.

    Note *Up to this point, the RNA has been protected from ribonucleases by the presence of guanidine isothiocyanate. The RNA is now no longer protected.*

15. For applications in which the salt concentration is important, wash the pellet with 75% (v/v) ethanol [DEPC-treated water (25%)]. Remove and discard the supernatant.

16. Dry the pellet under vacuum for 2–5 minutes. Do not over dry the sample because an over dry pellet will be difficult to resuspend.

17. Resuspend the RNA in 0.5–2 ml of DEPC-treated water. The A_{260}/A_{280} ratio should be ≥1.8 (see *Appendix I: Spectrophotometric Quantitation of RNA*).

Polyadenylated RNA [poly(A)$^+$] can now be isolated from the total RNA for use in subsequent cDNA synthesis and cloning, northern blot analysis, or translation studies.

For long-term storage, resuspend the RNA in DEPC-treated water, add 2 M sodium acetate to a final concentration of 0.25 M, and then add 2.5 volumes of 100% (v/v) ethanol. Store the RNA at –80°C.

Note *When ready to use the RNA after long-term storage in ethanol, repeat steps 14–17.*

Alternative Time-Saving Protocol for Isolating RNA

This time-saving protocol is an alternative protocol to *Isolating RNA* and is performed in 60–90 minutes.

1. Add 1.0 ml of 2 M sodium acetate (pH 4.0) to the sample. Mix the contents of the tube thoroughly by repeatedly inverting the tube.

2. Add 10.0 ml of phenol (pH 5.3-5.7) to the tube. Mix the contents thoroughly by inversion.

   Note *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.*

3. Add 2.0 ml of chloroform–isoamyl alcohol mixture to the homogenate. Cap the tube tightly and shake the tube vigorously for 10 seconds.

4. Transfer the homogenate to a 50-ml, thick-walled, round-bottom centrifuge tube.

5. Spin the tube in a centrifuge at 12,000 × *g* for 15 minutes at 5–10°C.

   Note *After centrifugation, two phases should be clearly visible. The upper aqueous phase contains the RNA. The lower phenol phase and interphase contain DNA and proteins.*

6. Taking care to avoid the interphase and the lower phase, transfer the upper, aqueous phase, which contains the RNA, to a sterile, RNase-free, 50-ml, thick-walled, round-bottom centrifuge tube. Discard the lower phenol phase, which contains proteins and DNA.

7. Add an equal volume of isopropanol to the tube that contains the aqueous phase and mix the contents by inversion.

8. Spin the tube in a centrifuge at 10,000 × *g* for 30 minutes at 5–10°C.

9. Remove and discard the supernatant.

   Note *Up to this point, the RNA has been protected from ribonucleases by the presence of guanidine isothiocyanate. The RNA is now no longer protected.*

10. Wash the pellet with 75% (v/v) ethanol [DEPC-treated water (25%)].

11. Remove and discard the supernatant. Dry the pellet under vacuum for 2–5 minutes. Do not over dry the sample because an over dry pellet will be difficult to resuspend.

12. Resuspend the RNA in 0.5–2 ml of DEPC-treated water. The A_{260}/A_{280} ratio should be ≥1.8 (see *Appendix I: Spectrophotometric Quantitation of RNA*).

TROUBLESHOOTING

Observation	Suggestion(s)
Interphase layer is not well resolved	Ensure that β-mercaptoethanol was added to the denaturing solution
	Ensure that chloroform–isoamyl alcohol solution was added to the sample
	Chloroform may have evaporated from the chloroform-isoamyl alcohol mixture, altering the ratio of chloroform to alcohol. When this occurs, phase separation is reduced. Add a few drops of chloroform to the extraction mixture, mix briefly by inversion, then recentrifuge the sample
	Ensure that sodium acetate was added to the sample. If sodium acetate was omitted from the extraction mixture, it may be added later to induce phase separation. Add the 1.0 ml of sodium acetate to the extraction mixture, mix by inversion, then recentrifuge the sample
	Remove the aqueous layer immediately after centrifugation, as the phases become less well resolved over time. Centrifuge the samples again for 5 minutes
Unable to resuspend the RNA pellet after precipitation	Increase the temperature of the final resuspension to 65°C for 5 minutes and increase the volume of the DEPC-treated water
	Vortex the pellet gently
	Add additional DEPC-treated water and allow the RNA pellet to dissolve for a longer time
Degraded RNA	The sample may be contaminated with RNase. Repeat the protocol, focusing strict attention on avoiding the introduction of RNases from hands and/or other contaminated surfaces, especially after step 14 of the protocol. At this stage the RNA pellet is particularly vulnerable to contaminating RNases
	Use DEPC-treated or radiation-sterilized plasticware.
	Introduce the tissue or tissue culture cells into solution D at the earliest possible moment to ensure inactivation of contaminating RNases
	If isolating the RNA from tissue, flash-freeze the tissue immediately after dissection from the animal. Some tissues, such as the spleen and pancreas, are very difficult to process without RNase degradation and must be flash-frozen immediately after isolation from the animal
	If isolating the RNA from cultured cells, remove cultured cells from the incubator at the last possible moment, process the cells rapidly once the medium is removed
The final optical density has an absorbance ratio (A_{260}/A_{280}) <1.8	The starting ratio of tissue or tissue culture cells to solution D is >100 mg/ml. Adjust the starting ratio of tissue or tissue culture cells to ~100 mg/ml
	During the phenol extraction, a portion of the middle and lower phases is removed along with the upper, aqueous phase that contains the RNA. **Carefully** avoid the middle and lower phases of the extraction while transferring the upper aqueous phase to a sterile, 50-ml polypropylene tube
	Avoid using DEPC-treated water in the spectrophotometric analysis as it skews the 260:280 absorbance ratio

(Table continues on the next page)

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The yield of RNA is lower than expected	Note that RNA yields vary depending on the tissues or cell lines employed
	The membranes of the tissue or tissue culture cells are not disrupted completely. Homogenize the tissue sample completely in solution D or disrupt the tissue culture cells by swirling or mixing thoroughly to release the nucleic acids
	The aqueous phase is not removed completely following extraction
	Some of the RNA pellet is decanted following the ethanol wash. Because residual ethanol can be removed from the RNA pellet via air-drying or under vacuum, **carefully** decant the ethanol wash to avoid discarding any of the RNA pellet
	The spectrophotometric measurement is inaccurate. Use ≥1µg of RNA in the spectrophotometric measurement
	The RNA pellet is resuspended incompletely. See *Unable to resuspend the RNA pellet after precipitation* in *Troubleshooting*
	Too great a mass of cultured cells is processed in a limiting volume, increasing the viscosity of the lytic mixture and causing significant loss of RNA to the phenol phase or the interphase layer
	Decrease the viscosity of the solution by passing the sample through an 18–21 gauge needle several times
DNA contamination	The lytic solution is too viscous and some DNA is left in the aqueous layer. To reduce and eliminate viscosity, see the recommendations in *The yield of RNA is lower than expected* in *Troubleshooting*
	Some of the interphase layer was removed with the aqueous layer. See *Interphase layer is not well resolved* in *Troubleshooting*
	See *Appendix V: Avoiding DNA Contamination*
PCR amplification of cDNA made from isolated RNA is smeary with excessive background bands	Use a smaller quantity of RNA as a template for cDNA (for 1 µg of total RNA reverse-transcribed into cDNA in a 50-µl volume, 1/5 of the cDNA reaction is enough to amplify extremely rare messages)

APPENDIX I: SPECTROPHOTOMETRIC QUANTITATION OF RNA

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

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

   Note *If the DEPC-treated water has pH<7, the quantitation should be performed in 5 mM Tris-HCl (pH 7.5) or TE buffer (see* Preparation of Media and Reagents*). 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 laboratory film (e.g., Parafilm® laboratory film) over the top of the cuvette and mix the sample well. Take the spectrophotometric reading. The conversion factor for RNA is 0.040 µg/µl per OD_{260} unit. For a reading of 0.10, calculate the concentration as follows:

   (Spec. reading A_{260}) × (Dilution factor) × (Conversion factor A_{260}) = Final concentration

   Example $(0.10)\times(500/5)\times(0.040\text{mg/ml}) = 0.4\text{mg/ml}$

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. Re-zero the spectrophotometer with the 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 1.8–2.0 are very pure. Lower ratios indicate 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 weights. 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), the agarose concentration should not exceed 0.8%. The following protocol is recommended for RNA of most sizes.

Protocol

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

   Note *The RNA can be dried completely without subsequent problem with resuspension since the loading buffer contains 48% formamide.*

2. In a flask, melt 1 g of agarose in 85 ml of deionized water.

3. Add 10 ml of 10× MOPS buffer (see *Preparation of Media and 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 the flask to mix the contents 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 the comb, and allow the gel to solidify in the fume hood.

4. While the gel solidifies, bring a water bath to boiling and prepare 5 µl of sample loading buffer for each sample. No more than 12 hours before use, prepare the loading buffer by mixing the components listed below:

   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 Media and Reagents*)

   Note *The loading buffer is not stable.* ***Do not*** *use the buffer more than 12 hours after preparation.*

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 the sample for 2 minutes, centrifuge the sample to collect condensation, and immediately load the sample onto the gel.

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

   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 under 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, with the zero point of the ruler 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 that 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 and immediately sinks to the bottom of the well. 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 soak with a 30–minute neutralization solution containing 0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl.

2. Prepare 500 ml of 10× SSC (see *Preparation of Media and 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).

3. Wet the Whatman 3MM paper with 10× SSC and drape the paper 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 3MM paper. Remove any bubbles trapped between the gel and the paper.

5. Cover the exposed areas of the Whatman 3MM paper that surround the gel with laboratory film (e.g., Parafilm laboratory film) 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 3MM 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 blot 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 3MM paper in 10× SSC and place them on top of the membrane. Remove any trapped bubbles.

9. Place 8–10 cm of absorbent material (paper towels or blotting material) on top of the Whatman 3MM paper and place 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. A balanced support 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 top two sheets of Whatman 3MM paper. Remove the laboratory film or plastic wrap and cut the Whatman 3MM 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 Whatman 3MM paper, upside down. Place the pile on a level surface and gently peel off the Whatman 3MM 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 the transfer efficiency by staining the gel in ethidium bromide and examining the gel 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.

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

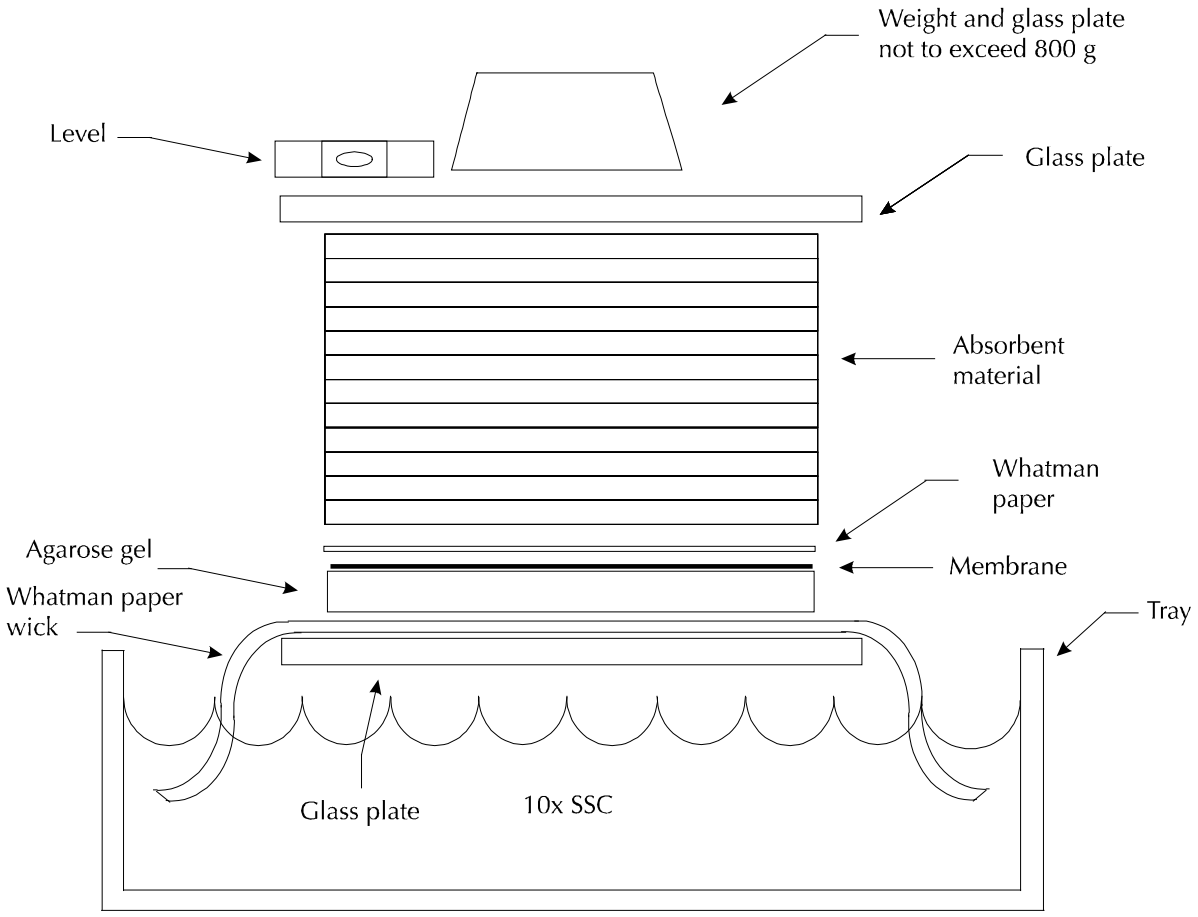

FIGURE 1 Capillary northern blot setup.

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 and hybridization solutions, which contain volume excluders, work very well in this application. If using a single-stranded DNA or RNA probe, 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 Agilent Illuminator nonradioactive detection system 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 the membrane on Whatman paper to remove most of the water.

2. Add the membrane to a container containing an appropriate amount of Agilent QuikHyb rapid hybridization solution or an appropriate amount of 6× SSC buffer, 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 for 2 hours with a standard prehybridization solution.

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

OLIGONUCLEOTIDES SHORTER THAN 18 BASES

$$T_m = 2°C(A + T) + 4°C(G + C)$$

OLIGONUCLEOTIDES 14 BASES AND LONGER (MAXIMUM OF 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 for 16–24 hours with another hybridization solution.

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

9. 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. Do not let the blot dry.

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

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.

APPENDIX V: AVOIDING DNA CONTAMINATION

Genomic DNA contamination of RNA isolated with the RNA Isolation Kit is minimal and should not cause a problem in most PCR amplifications. PCR amplification is so powerful, however, that contamination, by even 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:

- Choose primer sets that amplify the processed mRNA across the splice junctions, making the unprocessed genomic DNA an unamplifiable target.
- Choose primer sets that amplify cDNA made from processed mRNA and not made from an unprocessed genomic DNA template, which would be too large to PCR-amplify effectively.
- Choose primer sets to create genomic DNA and cDNA amplification products that are different sizes.
- Treat the RNA with RNase-free DNase I according to the following protocol:

1. 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 Media and Reagents*)
   1 U of DNase I (RNase-free)
   Bring the volume to 10 µl with DEPC-treated water

2. Incubate the reaction for 15 minutes at room temperature.

3. Add 1 µl of RNase-free 20 mM EDTA to the solution and heat the tube at 65°C for 10 minutes.

PREPARATION OF MEDIA AND REAGENTS

Notes *Prepare all solution with DEPC-treated water. Use a 0.1% (v/v) solution of DEPC in distilled water (dH_2O). Allow the DEPC-treated water to incubate overnight at room temperature and then autoclave the DEPC-treated water prior to use.*

If a solution contains Tris base, prepare the solution with DEPC-treated water and autoclave the solution ***before*** *adding Tris base. Autoclave the solution once again after the addition of Tris base to the solution.*

Denaturing Solution

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

10× 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)

Loading Dye

160 µl 10× MOPS Buffer
100 µl DEPC-treated water
100 µl ethidium bromide (10 mg/ml)[‖]
80 µl sterile glycerol
80 µl saturated bromophenol blue in sterile water

Note *Loading dye is stable if kept in a light-proof container. Always mix well before using. To make saturated bromophenol blue, add a small amount of bromophenol 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.*

[‖] If the RNA is to be transferred to a solid support (northern blot), use 5 mg/ml ethidium bromide solution.

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

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 10.0 N NaOH
Adjust volume to 1 liter with water

TE Buffer

5 mM Tris-HCl (pH 7.5)
1 mM EDTA

REFERENCES

1. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* 18(24):5294-9.
2. Chomczynski, P. and Sacchi, N. (1987) *Anal Biochem* 162(1):156-9.
3. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ENDNOTES

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

RNA Isolation Kit

Catalog #200345

QUICK-REFERENCE PROTOCOL

Homogenizing Animal Tissue

- Prepare solution D by adding 100 µl of β-mercaptoethanol to 14 ml of denaturing solution
- Quickly weigh 1 g of tissue and place the tissue sample in 10 ml of solution D
- Homogenize the tissue in 10 ml of solution D in a glass–PTFE homogenizer or a rotating blade homogenizer
- Proceed with *Isolating RNA* or the *Alternative Time-Saving Protocol for Isolating RNA*

Preparing Tissue Culture Cells Grown in Suspension

- Prepare solution D by adding 100 µl of β-mercaptoethanol to 14 ml of denaturing solution
- Gently pellet the cells by centrifugation and discard the supernatant
- Add 10 ml of solution D to the cell suspension and mix thoroughly
- Incubate the suspension for 1 minute at room temperature
- Transfer the cell suspension to a 50-ml polypropylene tube and proceed with *Isolating RNA* or the *Alternative Time-Saving Protocol for Isolating RNA*

Preparing Tissue Culture Cells Grown in Monolayer

- Prepare solution D by adding 100 µl of β-mercaptoethanol to 14 ml of denaturing solution
- Decant the tissue culture medium from the tissue culture plates
- Add 10 ml of solution D to a tissue culture plate and swirl the plate gently for 30 seconds
- Transfer solution D from the tissue culture plate to a second tissue culture plate and swirl the second plate for 30 seconds. Repeat this step for the remaining plates.
- Transfer the cell–solution D mixture to a 50-ml polypropylene tube and proceed with *Isolating RNA* or the *Alternative Time-Saving Protocol for Isolating RNA*

Isolating RNA

- Add 1.0 ml of 2 M sodium acetate (pH=4.0) to the tube and mix by inversion
- Add 10.0 ml of phenol (pH 5.3-5.7) to the tube and mix by inversion
- Add 2.0 ml of chloroform–isoamyl alcohol to the tube. Cap the tube tightly and shake the tube vigorously for 10 seconds
- Incubate the tube on ice for 15 minutes
- Transfer the sample to a prechilled, 50-ml, thick-walled, round-bottom centrifuge tube and spin in a centrifuge at 10,000 $\times$ *g* for 20 minutes at 4°C
- Transfer the aqueous phase, which contains the RNA, to a fresh centrifuge tube
- Add an equal volume of isopropanol to the tube and incubate the tube for ≥1 hour at –20°C to precipitate the RNA
- Spin the tube in a centrifuge at 10,000 $\times$ *g* for 20 minutes at 4°C
- Dissolve the pellet, which contains the RNA, in 3.0 ml of solution D
- Add 3.0 ml of isopropanol to the tube and incubate the tube for 1 hour at –20°C
- Spin the tube in a centrifuge at 10,000 $\times$ *g* for 10 minutes at 4°C. Discard the supernatant
- If the salt concentration is important, wash the pellet with 75% (v/v) ethanol
- Discard the supernatant and dry the pellet under vacuum for 2–5 minutes
- Resuspend the RNA in 0.5–2 ml of DEPC-treated water at an A_{260}/A_{280} ratio ≥1.8