### Integrity of RNA

The integrity and size distribution of total RNA can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see "A Guide to Analytical Gels" below). The respective ribosomal bands (Table 7) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present with an intensity approximately twice that of the 18S rRNA band (Figure 1). If the ribosomal bands in a given lane are not sharp, but appear as a smear of smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

**Table 7. Size of ribosomal RNAs from various sources**

| Source | rRNA | Size (kb) |
|---|---|---|
| *E. coli* | 16S | 1.5 |
| | 23S | 2.9 |
| *S.cerevisiae* | 18S | 2.0 |
| | 26S | 3.8 |
| Mouse | 18S | 1.9 |
| | 28S | 4.7 |
| Human | 18S | 1.9 |
| | 28S | 5.0 |

**Analysis of Total RNA**

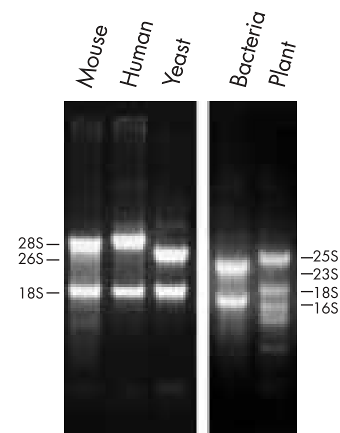


*Figure 1. Formaldehyde agarose gel of total RNA isolated from the indicated sources using RNeasy kits. 10 µg RNA was loaded per lane.*

# A Guide to Analytical Gels

## Principle of denaturing gel analysis

Formaldehyde agarose gels allow separation and identification of RNA based on charge migration. Unlike DNA, RNA has a high degree of secondary structure, making it necessary to use a denaturing gel. Formaldehyde in the gel disrupts secondary RNA structure so that RNA molecules can be separated by their charge migration.

In an electric field, nucleic acid molecules migrate towards the anode due to negatively charged phosphates along the backbone. The migration of denatured RNA molecules is determined by their size; however, the relationship between the fragment size and rate of migration is nonlinear, since larger fragments have a greater frictional drag and are less efficient at migrating through the gel.

Agarose gel analysis is the most commonly used method for analyzing RNA species, which generally correspond in size to the resolution range of an agarose gel. Small RNA fragments, such as tRNAs or 5S rRNAs, can be analyzed by polyacrylamide gel electrophoresis. Detailed information on all types of analytical gels can be found in current molecular biology manuals (3, 4). This section describes formaldehyde agarose gel electrophoresis.

## Preparing formaldehyde agarose gels for RNA analysis

The following protocol for formaldehyde agarose (FA) gel electrophoresis is routinely used at QIAGEN and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature of this protocol is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than provided for in conventional protocols

### Agarose

The concentration of agarose used for the gel determines the size range of RNA fragments that can be resolved. For most RNA species of interest, a concentration of 1.0–1.2% (w/v) agarose will give best results. For resolution of large mRNA species, it may be helpful to reduce the agarose concentration. For analysis of smaller mRNAs, the agarose concentration can be raised to 2%. With small RNA species, such as tRNAs or 5S rRNAs, polyacrylamide gel electrophoresis is recommended.

Tip Use ultrapure-quality agarose since impurities such as polysaccarides, salts, and proteins can affect the migration of RNA

* *Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.*

### Protocol 3. Pouring the gel

Protocol 3

1. Prepare enough 10x FA gel buffer to pour the gel and to make enough FA gel running buffer (see "Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA", page 94) to fill the electrophoresis tank.
2. Mix an appropriate amount of agarose, 10x FA gel buffer, and RNase-free water in a flask or bottle. To prepare FA gel of size 10 x 14 x 0.7 cm, mix:
   - 1.0–1.2 g agarose
   - 10 ml 10x FA gel buffer
   - Add RNase-free water to 100 ml

Tip If smaller or larger gels are needed, adjust the quantities of components proportionately.
The vessel should be no more than half full. Cover the vessel to minimize evaporation.

3. Heat the mixture in a microwave or boiling water bath, swirling the vessel occasionally until the agarose is dissolved.

Tip Ensure that the lid of the flask is loose to avoid buildup of pressure. Be careful not to let the agarose solution boil over as it becomes super-heated.

Tip If the volume of liquid reduces considerably during heating due to evaporation, make up to the original volume with RNase-free distilled water. This will ensure that the agarose concentration is correct.

4. Cool the agarose to 65–70°C in a water bath. Stir or swirl occasionally to prevent uneven cooling.
5. After cooling, add 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution.

Tip Formaldehyde is toxic. Use a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions when handling.

Tip Make sure that the solution has cooled sufficiently before adding formaldehyde and ethidium bromide. Formaldehyde is volatile and may evaporate if added to a solution that is too hot.

Tip Ethidium bromide in the gel allows visualization of the RNA with UV light. Ethidium bromide is toxic and a powerful mutagen. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals.

Tip Stock solutions of ethidium bromide (generally 10 mg/ml in water) should be stored at 2–8°C in a dark bottle or a bottle wrapped in aluminum foil.

protocol continues overleaf

**Protocol 3. Continued**

6. Pour the agarose solution onto the gel tray in a fume hood to a thickness of 3–5 mm. Insert the comb either immediately before or immediately after pouring. Let the gel set for at least 30 min.

Tip Ensure that there is enough space between the bottom of the comb and the gel tray (0.5–1.0 mm) to allow proper well formation and avoid sample leakage.

Tip Make sure that there are no air bubbles in the gel or trapped between the wells. Air bubbles can be carefully removed with a Pasteur pipet before the gel sets.

Tip Thicker gels can be used to increase the amount of sample volume that can be loaded. Thinner gels generally transfer better in northern blotting, but smaller sample volumes can be used.

Tip The thickness of the comb affects the sharpness of bands in the gel. A thinner comb gives sharper bands, but less sample can be loaded per well.

7. Leaving the comb in the gel, place the gel in the electrophoresis tank. Fill the tank with 1x FA gel running buffer.

Tip Add enough buffer to cover the gel with approximately 1 mm of liquid above the surface of the gel. If too much buffer is used, the electric current will flow through the buffer instead of the gel.

8. Carefully remove the comb from the gel. Prior to running, let the gel equilibrate in 1x FA gel running buffer for at least 30 min.

## Running and analyzing formaldehyde agarose gels for RNA analysis

**RNA loading buffer**

RNA loading buffer (see “Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA”, page 94) must be added to samples before loading them on a gel. The loading buffer serves three main purposes:

1. To denature the RNA sample prior to loading.
2. To increase the density of the samples to ensure that they sink into the wells on loading.
3. To add color to the samples through the use of dyes, facilitating loading and visualization on the gel while running.

A key feature of the concentrated RNA loading buffer described in “Formaldehyde Agarose Gel Electrophoresis Buffers for Analysis of RNA” (page 94) is that it allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols allow.

**Electrophoresis buffers**

RNA gels are run at a lower pH than DNA gels since RNA has a lower p$K_a$ than DNA. Furthermore, unlike DNA, RNA is susceptible to alkali cleavage at high pH. RNA gels should therefore be run at neutral pH. MOPS (3-[N-morpholino]propanesulfonic acid) is the most commonly used buffer for RNA gels due to its high buffering capacity at pH 7.0. Formaldehyde is included in the running buffer to keep the RNA denatured. Formaldehyde is also added to the agarose gel.

Tip Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol* and allowed to dry.

* *Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier’s instructions.*

## Protocol 4. Sample preparation for electrophoresis

Protocol 4

1. Add 1 volume of 5x RNA loading buffer to 4 volumes of RNA sample (for example, 5 µl of loading buffer and 20 µl of RNA) and mix.

Tip Samples should always be mixed with RNA loading buffer prior to loading on a gel.

Tip Do not use sample volumes close to the capacity of the wells as samples may spill over into adjacent wells during loading.

Tip Be sure that all samples have the same buffer composition. High salt concentrations will retard the migration of RNA molecules.

Tip Ensure that no ethanol is present in the samples, for example, carried over from purification procedures. Ethanol may cause samples to float out of the wells on loading.

2. To denature RNA, incubate for 3–5 min at 65°C. Chill on ice.

## Protocol 5. Electrophoresis

Protocol 5

1. Apply denatured samples to the wells of the gel. The gel should be submerged in electrophoresis buffer in the electrophoresis tank prior to loading.

Tip Prior to sample loading, remove air bubbles from the wells by rinsing them with electrophoresis buffer.

Tip Make sure that the entire gel is submerged in the FA gel running buffer.

Tip To load samples, insert the pipet tip deep into the well and expel the liquid slowly. Take care not to break the agarose with the pipet tip.

Tip Once samples are loaded, do not move the gel tray/tank as this may cause samples to float out of the wells.

Tip Be sure to include at least one lane of appropriate molecular-weight markers.

2. Connect the electrodes of the electrophoresis apparatus so that the RNA will migrate towards the anode or positive lead (usually red).

Tip The electrophoresis apparatus should always be covered to protect against electric shock.

Tip Run the gel in a fume hood to avoid exposure to formaldehyde fumes from the gel and running buffer.

3. Turn on the power supply, and run the gel at 5–7 V/cm until the bromophenol blue dye has migrated approximately 2/3 of the way through the gel.

Tip Avoid use of high voltages, which can cause trailing and smearing of RNA bands.

Tip Monitor the temperature of the buffer periodically during the run. High temperature can cause partial melting of the gel and distortion of the bands. If the buffer becomes significantly heated, reduce the voltage.

Tip For very long runs (e.g., overnight runs), use a pump to recycle the buffer.

#### Visual analysis of the gel

Ethidium bromide in the gel allows visualization of the RNA with UV light. Ethidium bromide is toxic and a powerful mutagen. Wear gloves and take appropriate safety precautions when handling. Use of nitrile gloves is recommended, as latex gloves may not provide full protection. After use, ethidium bromide solutions should be decontaminated as described in commonly used manuals (3, 4).

Tip Stock solutions of ethidium bromide (generally 10 mg/ml in water) should be stored at 2–8°C in a dark bottle or a bottle wrapped in aluminum foil.

#### Visualization

Ethidium bromide–RNA complexes display increased fluorescence compared to the uncomplexed dye in solution. This means that illumination of a stained gel under UV light (254–366 nm) allows bands of RNA to be visualized against a background of unbound dye. The gel image can be recorded by taking a Polaroid™ photograph or using a gel documentation system.

Tip UV light can damage the eyes and skin. Always wear suitable eye and face protection when working with a UV light source.

Tip UV light damages RNA. If RNA fragments are to be extracted from the gel, use a lower intensity UV source if possible, and minimize exposure of RNA to the UV light.

#### Analysis of total RNA

The integrity and size distribution of total RNA can be checked by observing the stained RNA. The respective ribosomal bands should appear as sharp bands on the stained gel (see **Table 7** and **Figure 1**, page 57). If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation. The 28S ribosomal RNA band should be present at approximately twice the intensity of the 18S rRNA band. Since the 28S rRNA is more labile than the 18S rRNA, equal intensities of the two bands generally indicates that some degradation has occurred.

## RNA analysis by northern blotting

### Principle of RNA blotting

After separating RNA molecules based on charge migration in a denaturing gel, RNA molecules in the gel are transferred to a nylon or nitrocellulose membrane by capillary transfer. The RNA of interest can then be identified by hybridization to radioactive or chemoluminescent probes and visualized by autoradiography or photography.

Since DNA blotting is commonly referred to as “Southern blotting”, after its inventor E.M. Southern, the term “northern blotting” was coined for this analogous RNA-blotting process.

### The northern blotting procedure

The following protocol for northern blotting is routinely used at QIAGEN. This procedure is intended for use with a standard formaldehyde agarose gel, prepared and run as previously described (pages 57–61).

### Blotting membrane

Northern blotting is generally carried out by immobilization of the RNA on nylon or nitrocellulose membranes. Positively charged nylon membranes are generally recommended over nitrocellulose because of their greater strength and ease of handling.

Tip Always wear gloves while working with blotting membranes. Handle membranes carefully by the edges or using clean blunt-ended forceps.

### Transfer buffer

Formaldehyde agarose RNA gels are generally blotted using a high-salt buffer such as 20x SSC (see “Northern Transfer Solution”, page 95). Prepare 1.2 liters of 20x SSC. For larger RNA gels (>100 ml volume), use 2.4 liters of 20x SSC.

Tip Save 200 ml of the 20x SSC solution, and dilute it twofold to make 10x SSC for soaking the gel and washing the blot after transfer.

### Equipment required

- Whatman® 3MM filter paper
- Paper towels, a stack of approximately 15–20 cm
- Plastic wrap
- Two glass or Plexiglas® plates
- Buffer tray (e.g., glass casserole dish) capable of holding 1–2 liters of buffer
- Flat weight, approximately 1 kg
- RNase-free water (200 ml)
- 0.05 M NaOH (200 ml)

Protocol 6

## Protocol 6. Presoaking filter paper and blotting membrane

1. Cut one sheet of nylon membrane and two sheets of Whatman 3MM paper about 1 mm larger than the gel on each edge.
2. Cut two lengths of Whatman paper wider than the gel, long enough to fit under the gel and reach to the bottom of the dish on either side (see Figure 2).
3. Wet the nylon membrane in water. Then soak the Whatman paper and nylon membrane in 20x SSC for 1–2 min.

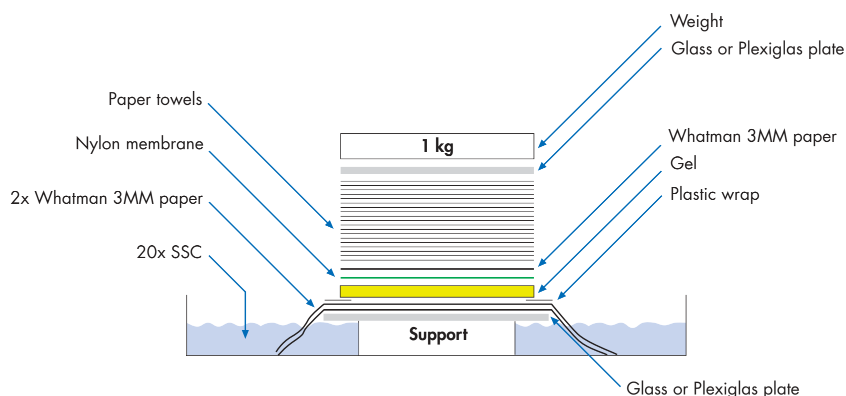


*Figure 2. Northern blot setup.*

Protocol 7

## Protocol 7. Capillary transfer

1. Fill the buffer tray with 1 liter 20x SSC. Place a glass or Plexiglas plate across the tray or on top of a support (Figure 2).
2. Place the two lengths of presoaked filter paper over the glass or Plexiglas plate so that the ends contact the bottom of the tray (Figure 2). Remove any air bubbles between the sheets of filter paper and the plate by rolling a pipet several times back and forth over the surface.
3. Immediately after gel electrophoresis, soak the gel for 10 min, with gentle shaking, in 200 ml RNase-free water and then for 15 min in 200 ml 0.05 M NaOH. Finally, soak the gel for 10 min in 200 ml 10x SSC to neutralize the NaOH.

Tip Dilute 20x SSC twofold to make 10x SSC.

Tip The gel contains formaldehyde to denature the RNA. Formaldehyde is toxic. Use a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions.

4. Position the gel upside-down on the filter paper covering the plate.
5. Place a sheet of plastic wrap over the gel. Use a sheet large enough to cover the surface of the filter paper on the glass or Plexiglas plate. Using a clean scalpel or razor blade, carefully cut the plastic wrap around the gel. Remove the piece over the gel so that the remaining plastic wrap surrounds the gel. This ensures that the transfer buffer moves only through the gel and not around it.
6. Place the presoaked nylon membrane on top of the gel so that it covers the entire surface (Figure 2). Do not move the nylon membrane once it has been placed on the gel. Remove any air bubbles between the membrane and the gel by gently rolling a pipet several times back and forth over the surface.
7. Place the two presoaked sheets of Whatman 3MM paper on top of the nylon membrane (Figure 2). Again, remove any air bubbles by gently rolling a pipet several times back and forth over the surface.
8. Place a 15–20 cm stack of dry paper towels on top of the filter paper (Figure 2).

protocol continues overleaf

**Protocol 7. Continued**

*Tip* Make sure that the plastic wrap around the gel prevents contact of the paper towels with the transfer buffer and the wet filter paper under the gel. Ensure that the towels do not droop over since they can cause liquid to flow around the gel instead of through it.

**9.** Place a second glass or Plexiglas plate on top of the paper towels. Place the 1 kg weight on top of the plate (**Figure 2**).

**10.** Let the transfer proceed for 12–18 h.

*Tip* Remove the wet paper towels and replace them with dry ones at least once during the transfer. If necessary, add more transfer buffer to the buffer tray.

*Tip* The gel contains formaldehyde which will diffuse out of the gel during transfer. Formaldehyde is toxic. Perform the transfer in a fume hood to avoid inhalation. Wear gloves and take appropriate safety precautions when handling.

## Protocol 8. Fixing the RNA to the blot



**1.** After the transfer is complete, remove the weight, paper towels, and the two sheets of filter paper. Turn over the gel and the nylon membrane together, and lay them, gel-side up, on a dry sheet of filter paper. Mark the positions of the gel lanes on the membrane using a ball-point pen or a soft-lead pencil. Peel the gel from the membrane and discard it.

*Tip* Make sure to mark the gel lanes before removing the gel from the nylon membrane! Without this marking, you won't be able to tell which lane is which.

*Tip* Most of the formaldehyde in the gel transfers into the paper towels and the upper sheets of filter paper. Dispose of them according to your institution's waste-disposal guidelines.

**2.** Wash the nylon membrane for 1 min in 100–200 ml 10x SSC.

*Tip* Dilute 20x SSC twofold to make 10x SSC. This wash step is critical to remove any agarose that adheres to the blot.

**3.** Fix the RNA to the blot by baking (step 3A) or UV-crosslinking (step 3B).

*Tip* UV-crosslinking generally gives better results and enhanced sensitivity compared to baking. However, proper crosslinking requires prior optimization of the system.

**3A.** To fix the RNA by baking, first let the blot air-dry on a dry sheet of filter paper, then place between two sheets of filter paper. Bake for 30 min to 2 h at 80°C in a vacuum oven.

**3B.** To fix the RNA by UV-crosslinking, take the damp blot and expose the side with the RNA to UV irradiation (e.g., with a UV transilluminator) for a determined length of time.

*Tip* To determine the proper conditions for UV irradiation, the system must first be empirically tested and optimized. To do this, take an RNA blot with several lanes containing identical RNA samples. Cut the blot into separate strips for each lane, and irradiate each for different times, varying from 0.5 to 5 min. After hybridization, determine which time gives the optimal signal intensity. Be sure to use the same conditions (UV wavelength, distance from UV source) for each experiment. In addition, the system should be routinely calibrated to determine that the intensity of the UV irradiation remains unchanged.

*Tip* UV light can damage the eyes and skin. Always wear suitable eye and face protection.

**4.** If the blot is not to be used immediately, store it at 4°C, wrapped in plastic wrap.