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Protocol

Polyacrylamide Gel Electrophoresis of RNA

Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen

INTRODUCTION

Perhaps the most important and certainly the most often used technique in RNA analysis is gel electrophoresis. This technique is generally applicable for RNA detection, quantification, purification by size, and quality assessment. Because RNAs are negatively charged, they migrate toward the anode in the presence of electric current. The gel acts as a sieve to selectively impede the migration of the RNA in proportion to its mass, given that its mass is generally proportional to its charge. Because mass is approximately related to chain length, the length of an RNA is more generally determined by its migration. In addition, topology (i.e., circularity) can affect migration, making RNAs appear longer on the gel than they actually are. Gels are used in a wide variety of techniques, including Northern blotting, primer extension, footprinting, and analyzing processing reactions. They are invaluable as preparative and fractionating tools. There are two common types of gel: polyacrylamide and agarose. For most applications, denaturing acrylamide gels are most appropriate. These gels are extremely versatile and can resolve RNAs from ~600 to ≤ 20 nucleotides (nt). In certain circumstances, e.g., resolving different conformers of RNAs or RNA-protein complexes, native gels are appropriate. The only disadvantage to acrylamide gels is that they are not suitable for analyzing large RNAs (≥ 600 nt); for such applications, agarose gels are preferred. This protocol describes how to prepare, load, and run polyacrylamide gels for RNA analysis.

RELATED INFORMATION

In this protocol, we describe one specific example of a denaturing polyacrylamide gel. However, this example can be easily extrapolated to any size gel. In this regard, we prefer to run the same size (large) gels for all applications, whereas other investigators prefer to run smaller gels. For experiments requiring separation of large molecules (≥ 600 nt), a method for use of agarose gels is described in **Nondenaturing Agarose Gel Electrophoresis of RNA** (Rio et al. 2010). It is imperative that good practices are used to avoid misshapen, streaky, or otherwise ugly gels. Because good habits are essential for obtaining good results, we recommend that the beginning investigator practice running gels before investing valuable samples.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with **<!**, and recipes for reagents marked with **<R>**.

Perform all procedures under RNase-free conditions, using RNase-free glassware and other equipment, and prepare all reagents with RNase-free H_2O .

Reagents

<R>Acrylamide stock for RNA gels (40%)

Adapted from *RNA: A Laboratory Manual*, by Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen. CSHL Press, Cold Spring Harbor, NY, USA, 2010.

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We recommend purchasing premade solutions of acrylamide:bisacrylamide (BioRad Amresco) to avoid exposure to dry chemicals.

<!>Ammonium persulfate (10%, w/v, freshly prepared)

H₂O, RNase-free

<!>N,N,N',N'-Tetramethylethylenediamine (TEMED), electrophoresis grade

<R>RNA gel-loading buffer (1.5X)

For additional information on loading buffers and dyes, see individual protocols regarding sample preparation and appropriate loading dyes.

RNA samples

For large-scale purification (e.g., from in vitro transcription reactions), pellets contain high concentrations of nucleotides and salt. Do not overload the gel with these samples; it is advisable to run such samples in multiple lanes.

<R>TBE electrophoresis buffer (10X)

<!>Urea, ultrapure

Equipment

Beaker (200 mL), baked

Bench paper

Binder clips

Combs and spacers (0.4 mm)

If running preparative gels, use thicker spacers and combs, e.g., 0.8 mm.

Equipment for filtering acrylamide solutions (if using homemade solutions; see Step 3.iii)

This includes Whatman No. 1 paper, a Buchner funnel, a 250-mL side-arm Erlenmeyer flask or a 0.45-μm bottle-top filter, and a rubber stopper for the flask.

Ethanol or glass cleaner (e.g., Windex) for cleaning gel plates

Gel drier or vacuum drier

Gel-loading tips

Gel plates (20 × 40 cm)

We run 20- × 40-cm gels for almost every acrylamide application because these large gels are very versatile and afford maximal resolution for RNAs of any size ≤600 nt.

Gloves

Heat block preset to 90°C-95°C

High-voltage power supplies

Ice

Large laboratory tissues (e.g., Kimwipes)

Micropipettor (e.g. Pipetman)

Pasteur pipette or syringe for rinsing wells (see Steps 6.ii and 6.iv)

A squirt bottle can be used as an alternative in Step 6.ii.

Phosphorimaging or autoradiography equipment

Plastic wrap

Razor blades

Safety glasses

Silicone grease (optional; see Step 8.i)

Siliconizing agent (e.g., Rainex)

Spatula, thin (dedicated for prying apart gel plates)

Squirt bottle

Stir bar, baked

Syringe (60 mL) for pouring gel

Thermometer (optional; see Step 8.i)

Tray (e.g., cafeteria-style)

Vacuum system for degassing (if using homemade acrylamide solutions; see Step 3.iii)

Vertical gel box

Water bath preset to 60°C

Whatman 3MM paper

Yellow tape (Scotch 3M electrical, 1.5-in.)

METHOD

1. Before beginning, assemble all equipment and solutions.
2. Prepare the gel plates; gel plates must be cleaned before each use.
 - i. For first-time use, wash with soap and water, then dry. Mark the outside of the plate and then clean the inside surface with glass cleaner or ethanol. If there is any particulate material or dried acrylamide evident, scrape clean with a new single-edge razor blade, but do not scratch the glass plates.
 - ii. Treat the inside of the shorter plate with a siliconizing agent or preferably a commercial substitute such as Rainex. Apply Rainex to the plate with a paper towel or large laboratory tissue. Cover evenly but do not allow Rainex to get on the edges or the other side of the plate. Air-dry and wipe clean with laboratory tissues. Rinse with water and dry for use.

The water should bead up, indicating that the surface has been properly treated. This treatment should last for many gel runs. Re-treat when the water fails to bead on the surface of the plate.
3. Prepare the gel solutions.

The following protocol is for a 6% gel. This is enough gel solution to make a 20- × 40-cm × 0.4-mm-thick gel with enough left to use in the event that some solution gets lost or is required to be used as refill after any leakage. Adjust as necessary for other percentages.

 - i. For 100 mL, weigh out 48 g of urea (electrophoresis grade) in a 200-mL baked beaker; add 15 mL of acrylamide stock for RNA gels (40%), 10 mL of 10X TBE electrophoresis buffer, and water to 100 mL. Stir to dissolve with a baked stir bar.

The final concentration of urea is 8 M.
 - ii. Heat briefly in a 60°C water bath.

This helps the dissolving process, but do not heat for long because the solution should be at room temperature for the polymerization step.
 - iii. If you prepare your own acrylamide solutions, filter the gel mix through three layers of Whatman No. 1 paper using a Buchner funnel and a 250-mL side-arm Erlenmeyer flask or a 0.45-µm bottle-top filter. Remove the funnel, place a rubber stopper over the top of the flask, and degas briefly under vacuum.
4. Assemble the plates (Fig. 1) as follows:
 - i. Place spacers on the edges of the inside surface of the long plate. Cover with a second plate, with the treated side down; be careful not to move the spacers. Hold in place with a binder clip on one side (Fig. 1A).
 - ii. Tape the unclipped side and part of the bottom with yellow tape; use the nonsharp side of a single-edge razor to smooth the tape. (Razor blades are the most useful for cutting tape.) If the small plate does not have “ears,” extend the tape over gel spacer tabs. Clip the taped side one-third of the way up from the bottom. Make sure that the end of the clip is positioned over the tape and spacer.
 - iii. Unclip the other side and tape the plate as described above. Tape the bottom of the plates overlapping a few inches up the sides. Check to ensure that the comb fits securely (Fig. 1B).
5. Pour the gel (see Fig. 1C) as follows.

Have everything ready for pouring because the solution will polymerize quickly; the higher percentage the gel, the more quickly it will polymerize. Use a cafeteria tray or its equivalent to catch any spilled or overflow acrylamide solution.

 - i. Add 900 µL of 10% ammonium persulfate and 80 µL of TEMED and mix briefly with a stir bar. Fill a 60-mL syringe with the gel solution.
 - ii. Tip the assembled plates up and balance on one bottom corner. Using the syringe, slowly and steadily run the gel solution down the side on which the plates are balanced. As the solution reaches the bottom corner, rotate the bottom plates so that the solution spreads across the bottom to the other corner. As you continue to supply solution, slowly move the plates to a more level (horizontal) position so that the gel fills the plates from bottom to top.

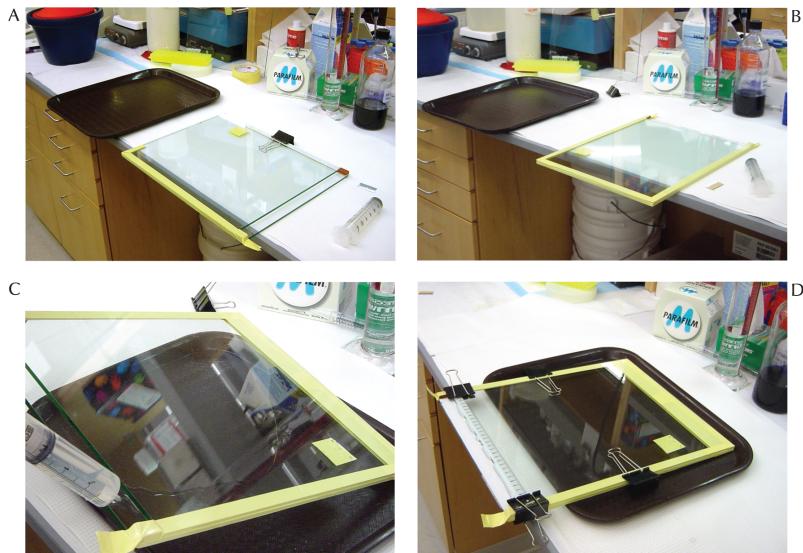


FIGURE 1. Preparing the gel plates and pouring the gel. (A) Partially taped plates. (B) Fully taped plates. (C) Pouring the gel. (D) Clamped comb in place.

- iii. When the solution reaches the top of the short plate (with no bubbles), lay the gel down in the tray with the top resting on the edge. Insert the comb and clamp both top corners so that the comb is held tightly between the two plates (Fig. 1D).

If bubbles form around the wells, remove the comb and try again to ensure that the acrylamide solution covers the wells.

If the fit is not tight, a thin film of polymerized acrylamide will form between the wells and the plates and will interfere with sample loading in Step 7.

- iv. Leave the remainder of the gel solution in the beaker to check for polymerization. Once the solution has completely polymerized (~15-20 min), leave the gel for another 5-10 min. Make sure that the partitions between the teeth of the comb are completely formed.

As polymerization proceeds, the refractive index of the gel changes and this can be visualized as a "Schlieren line" near the edge of the gel plate and comb. This effect is a result of a change in refractive index between the polymerized and unpolymerized acrylamide.

6. Set up the gel as follows once it is polymerized:

- i. Remove all clips and the tape from the bottom edge (or slice with a razor blade) and rinse the top of the gel around the comb using a squirt bottle filled with water.

- ii. Remove the comb and rinse the wells with water (using a squirt bottle, syringe, or Pasteur pipette).

- iii. Attach the gel plates to the gel apparatus, with the short side in. Clamp in place with equal pressure on both sides.

- iv. Add TBE electrophoresis buffer to the top and bottom chambers. Use a Pasteur pipette or syringe to rinse the wells again, this time with TBE; make sure that there are no air bubbles in the wells and that no air bubbles are trapped under the bottom of the gel.

- v. Close the lids, attach the electrodes (red on the bottom, black on the top), and prerun the gel at 45 mA for 20-45 min.

Prerunning removes excess persulfate and eliminates hyperfocusing. Longer preruns (45 min) are recommended for analyzing short RNAs of ≤ 50 nt.

7. Load the gel as follows after the prerun is completed:

- i. Turn the power supply off.

- ii. Add RNA gel-loading buffer to the RNA samples, heat the samples at 90°C-95°C, and chill on ice.
 - iii. Rinse the wells again to disperse the urea (which tends to diffuse out of the gel and remain in the bottom of the wells).

This will allow the sample to settle with as little upward spread as possible.

- iv. Using a micropipettor, load the RNA samples quickly so that the wells remain free of urea. Avoid creating air bubbles, which will disturb the samples.

Thin gel-loading flat micropipette tips that fit between the 0.4-mm spacers and reach the bottom of the well are available. In general, load minimal volumes of ≤10 µL per 0.8-cm-wide well with a 0.4-mm-thick gel. Adjust the loading volume according to thickness and width of the well. For preparative applications, run thicker gels.

8. Run the gel:

- i. For a 20-cm-wide × 40-cm-long × 0.4-mm-thick gel, run the gel either at constant current (45 mA) or constant voltage (1600 V).

It is important not to run the gels too fast. Haste makes waste! A thermometer to monitor heating can be attached to the front plate with silicone grease. The temperature must remain under ~58°C. Increasing the current or the voltage runs the risk of overheating the gel or even cracking the gel plates. Overheating or running too fast can greatly distort the appearance of bands and accentuates any irregularities in the gel matrix.

High-percentage gels must run at constant current to avoid overheating. The millamps are determined by the size and percentage of the gel.

- ii. Run the appropriate gel distance to optimize the resolution of the bands of interest.

The tracking dyes in the loading buffers (xylene cyanol and bromophenol blue) ALWAYS run to the same position, but depending on the percentage gel, they comigrate with different-sized nucleic acids. Gels are highly reproducible when prepared and run under the same conditions; therefore, it is advisable to become accustomed to the behavior of the specific gel you use.

See Troubleshooting.

9. Disassemble the gel:

- i. Turn the power supply off and drain the buffer. If the RNA sample run was radioactive, the bottom buffer in particular may be radioactive and should be handled accordingly.

- ii. Detach the plates and lay them down on bench paper, with the large plate down. Remove the yellow tape, either with a razor blade or by pulling it off, if possible. Slide the spacers out of the sides.

- iii. Using a thin spatula (dedicated to this purpose only), pry the top plate up, starting from a corner. Do this slowly until it is clear that the gel remains on the bottom plate. Wear safety glasses in the event that the corner of the glass chips.

See Troubleshooting.

- iv. Trim a piece of Whatman 3MM paper to the size of the gel and carefully lay it over the gel.

Smooth the paper with a gloved hand to ensure uniform contact with the gel. Pick up the paper (and gel) and lay them gel side up on clean bench paper.

Gels can be covered by plastic wrap and exposed to X-ray film at -80°C without drying. However, to prevent damage to phosphor screens, drying is recommended.

- v. Cover the gel with plastic wrap and dry at 80°C using a vacuum-driven gel drier.

Make sure that the gel is completely dry before opening the drier. If the drier is opened too soon, the gel may crack or shrivel.

See Troubleshooting.

10. Expose the gel to the PhosphorImager or perform autoradiography.

Gels dried without fixing and washing in methanol-acetic acid remain tacky after drying because of the urea. Leaving them on the plastic wrap will help to protect the phosphor screen or prevent the gel from sticking to the film.

See Troubleshooting.

TROUBLESHOOTING

Problem: "Smiling" of marker dyes is observed.

[Step 8.ii]

Solution: "Smiling" of marker dyes is caused by uneven heat distribution when using a gel apparatus without a metal backing plate. Other causes are improper taping or clamping of plates, a bowed plate, a bad spacer (unmatched), and running the gel too fast. To avoid heat-related problems, run the gel more slowly or use a gel apparatus with a metal diffuser plate.

Problem: The tracking dye is hyperfocused.

[Step 8.ii]

Solution: A hyperfocused tracking dye indicates that the gel was not prerun for enough time. Prerun gels adequately, making sure that running conditions are the same as those of the prerun.

Problem: Sample is trapped in the wells.

[Step 8.ii]

Solution: If the sample is trapped in the wells, this may be the result of inadequate resuspension of the sample, aggregates in the sample, or inadequate denaturation. Make sure that the sample is well dissolved and not overconcentrated. Denature properly.

Problem: The gel sticks to both plates.

[Step 9.iii]

Solution: This can be caused by inadequate "waterproofing" of the short plate or overheating during the run. Re-treat the top plate with Rainex and make sure that run conditions are correct.

Problem: The gel cracks after drying.

[Step 9.v]

Solution: If this occurs, the gel was not completely dry. Dry gels for a longer amount of time and make sure that the gel drier vacuum is sealed.

Problem: Lanes are distorted.

[Step 10]

Solution: Distortion of lanes is caused by inappropriate pulling of combs or acrylamide in wells, salt in samples, overloading the gel, or bubbles in the gel. Consider the following:

1. Make sure that the tops of the wells are wet when pulling the comb and rinse sample wells adequately.
2. For the problem of salt in the sample, reprecipitate with ethanol.
3. If overloading is the culprit, reduce the amount of sample loaded.

Problem: Streaks of radioactivity are seen.

[Step 10]

Solution: Streaking of radioactivity down the lanes can be the result of several of the above situations, including those mentioned in "smiling" of marker dyes, sample trapping in the wells and lane distortion. See the solutions to these problems above.

REFERENCES

Rio DC, Ares M Jr, Hannon GJ, Nilsen TW. 2010. Nondenaturing agarose gel electrophoresis of RNA. *Cold Spring Harb Protoc* (this issue). doi: 10.1101/pdb.prot5445.