Chapter 5

Polyacrylamide Gel Electrophoresis for Purification of Large Amounts of RNA

Mélanie Meyer and Benoît Masquida

Abstract

Polyacrylamide gel electrophoresis (PAGE) constitutes a powerful technique for the efficient purification of RNA molecules dedicated to applications that require high purity levels. PAGE allows for the fractionation of RNA obtained from cell extracts, chemical or enzymatic synthesis, or modification experiments. Native or denaturing conditions can be chosen for analytical or preparative-scale separations and the nucleotide resolution can be tuned by changing the percentage and reticulation of the gel material. In this protocol, we focus on the preparation of milligram-scale amounts of ~200 nucleotides (nt) RNA molecules that were used in subsequent crystallization experiments.

Key words Gel electrophoresis, RNA purification, In vitro transcription, RNA degradation

1 Introduction

In the landscape of techniques used for RNA purification, polyacrylamide gel electrophoresis (PAGE) remains a method of choice. It has been in use for >50 years and has been extensively described, especially in the laboratory handbook from Sambrook and Russell [1]. As stated there, the gel material is obtained by a radical chain reaction leading to the polymerization of vinyl groups from acrylamide monomers. The resulting long chains are simultaneously cross-linked together by adding bisacrylamide moieties. To separate macromolecules, the acrylamide/bisacrylamide ratio, as well as the concentration of polyacrylamide in the gel material (typically between 5 and 20 %), can be varied to modify the gel reticulation. These properties confer to the polyacrylamide gel material a resolving power and a loading capacity that hold it as a fair competitor of other purification process, such as gel filtration or ion-exchange chromatography [2]. Moreover, there is, a priori, no need for RNA engineering unlike other RNA purification strategies that are based on the adjunction of affinity tags in order to purify the RNA of interest under native conditions (*see* [3] for

Eric Ennifar (ed.), *Nucleic Acid Crystallography: Methods and Protocols*, Methods in Molecular Biology, vol. 1320,
DOI 10.1007/978-1-4939-2763-0_5,

review). In fact, PAGE can also be performed under native or denaturing conditions at different temperatures, which offers enough diversity to optimize purification protocols. Likewise, temperature gradient gel electrophoresis (TGGE [4, 5]) can in principle also be brought into play in order to identify the best conditions for the purification of a given RNA. A handful of different RNAs can be purified simultaneously by one user, which enables their parallel study in subsequent experiments.

In this protocol, we focus on the purification of RNAs produced by large-scale in vitro transcription reactions of up to 10 mL volumes [6, 7], which were further used for crystallization experiments [8, 9]. In this context, several dozens of different RNA constructs usually need to be studied to yield suitable crystals. The systematic use of PAGE allows for both the purification and the conformational study of these RNAs under denaturing or native conditions, respectively. PAGE is thus a powerful method to facilitate the selection of constructs with satisfying refolding properties, a prerequisite to obtain crystals. Moreover, when discrete conformations are resolved, purification under native conditions can also be achieved to overcome the coexistence of alternative conformations in the sample mix, typically resulting from the application of RNA refolding protocols based on heating/cooling steps [10, 11]. This chapter presents the different steps from the assembly of the apparatus to harvesting of the purified RNA.

2 Materials

Prepare all solutions using ultrapure water (optionally DEPC treated). Except when they contain acrylamide, solutions are filtered through a 0.22 μm sterile filter and kept at room temperature, unless otherwise specified. Acrylamide and bisacrylamide solutions are commercially available in order to prepare gel solutions with a user-defined reticulation. However, acrylamide/bisacrylamide reticulation ratios of 19:1, 29:1, or 37.5:1 (available from Carl Roth, Karlsruhe, Germany, or Bio-Rad, Hercules, CA, USA) optimized for macromolecule separation of increasing molecular weight, respectively, are available as premix gel solutions (*see* **Note 1**).

2.1 Gel Apparatus Components

1. Two glass plates, with dimensions 35×40 cm^2 and 35×42 cm^2.
2. 0.2 cm thick gel spacers.
3. Four paper clips for holding the glass plates and spacers together.
4. (Optional) Water-soluble siliconizing fluid (silane- or siloxane-containing solutions from Thermo Fisher Scientific, Waltham, MA, USA, or Sigma-Aldrich, Saint Louis, MI, USA) (*see* **Note 2**).
5. Adhesive tape (5 cm wide roll brown tape).

6. 0.2 cm thick comb forming loading wells of at least 4 mL.
7. PAGE gel apparatus (i.e., Model S2 Sequencing Gel Electrophoresis Apparatus—Life Technologies, Carlsbad, CA, USA) (*see* **Note 3**).
8. Power supply for gel apparatus.

2.2 Denaturing PAGE Components

1. 8 M Urea stock solution.
2. 40 % Acrylamide/bisacrylamide (19:1) stock solution (i.e., Rotiphorese® Gel 40 (19:1)—Carl Roth, Karlsruhe, Germany).
3. Tris–borate–EDTA buffer (TBE 10×): 216 g of Tris base, 55 g of boric acid, 40 mL of 0.5 M EDTA pH 8.0, adjust with water up to 1 L.
4. APS 10 % stock solution: Weigh 1 g of ammonium peroxodisulfate to dissolve in 10 mL of water (*see* **Note 4**). Store at 4 °C.
5. TEMED: *N,N,N′,N′*-tetramethylethylenediamine. Store at 4 °C.

2.3 Sample Preparation and Migration

1. Absolute ethanol. Store at −20 °C.
2. Urea loading buffer 2× with or without tracking dyes: 8 M urea, 0.025 % xylene cyanol, 0.025 % bromophenol blue.

2.4 RNA Recovery

1. Transparent plastic film.
2. Silica plate (several adjoined thin-layer chromatography (TLC) plates).
3. UV lamp 254/302 nm with light emitted from above the bench.
4. Sterile scalpel blades.
5. 10 mL Terumo® syringes.
6. 50 mL BD Falcon™ conical tubes.
7. Roller mixer.
8. Analytical 0.2 μm filter units for 50 mL tubes (Thermo Scientific Nalgene, Rochester, NY, USA).
9. 3 M Ammonium acetate pH 5.2.
10. Ultrapure (>99.5 %) absolute ethanol for analysis. Store at −20 °C.
11. UV spectrophotometer, Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) or equivalent.

3 Methods

3.1 Preparation, Polymerization, Casting, and Mounting of the Gel

1. Prepare a 25 % acrylamide/bisacrylamide/urea (19:1:8 M) stock gel solution, by dissolving 480 g of urea in 625 mL of acrylamide/bisacrylamide 40 % (19:1). Make up the volume to 1 L by addition of ultrapure water (*see* **Note 5**).

2. Thoroughly clean the glass plates first with water and then with ethanol.
3. (Optional) Siliconize one glass plate to ease taking down the gel at the end of the migration (*see* **Note 6**). Pour 5 mL of siliconizing solution onto the clean plate laid under the hood and spread over the whole plate using a paper towel. Allow the remaining liquid to evaporate thoroughly before cleaning the plate a second time.
4. Assemble glass plates with spacers by aligning the bottom of the glass plates with one extremity of each spacer. Clip the gel mold, and seal its sides and bottom with tape. Make sure that the tape is not punctured in the lower corners of the gel mold to avoid leakage of the acrylamide solution upon casting.
5. Prepare a gel solution (*see* **Note 7**) by mixing a volume of the 25 % stock gel solution from **step 1** required to obtain the target acrylamide/bisacrylamide percentage with the volume of 8 M urea solution required to prepare the amount of solution for the mold from **step 4**. Take into account that the final gel solution should contain 1× or 0.5× final TBE concentration. Typically, to purify RNA of 200 nucleotides we prepare an 8 % polyacrylamide solution.
6. (Optional) Degas the solution under vacuum in a side-arm flask at least fourfold larger than the volume of acrylamide solution to avoid overflow through the side arm.
7. Pour the gel solution in a clean beaker tenfold larger than the volume of the gel solution. Trigger polymerization by adding 528 µL of freshly prepared 10 % APS solution followed by 50 µL of TEMED per 100 mL of gel solution. Mix thoroughly by shaking for few seconds and immediately cast the gel within the glass mold. Slightly tilt the mold to favor the entrance of the gel solution on one side to avoid trapping air bubbles.
8. When the mold is full, lay it over the bench and gently insert the combs.
9. Wait until the gel solution remaining in the beaker is fully polymerized, which indicates that the polymerization within the gel mold is complete. Do not discard non-polymerized gel solutions (*see* **Note 8**).
10. Remove paper clips, tape, and combs.
11. Mount the gel onto the electrophoresis apparatus.
12. Add 1× or 0.5× TBE buffer to the chambers.
13. Wash the urea out of the wells with TBE buffer from the top chamber using a 1 mL pipet.
14. Pre-run the gel for half an hour at 50 W to reach a temperature around 50 °C.

3.2 Sample Preparation and RNA Migration

1. Precipitate the large-scale (5–10 mL) transcription reaction by addition of 2.5 volumes of cold pure ethanol.
2. Spin the tubes containing the crude RNA at 20,000 × *g* for 20 min at 4 °C in 50 mL BD Falcon™ conical tubes (*see* **Note 9**).
3. Discard the supernatant and resuspend the pellet in 1 mL of water (*see* **Note 10**).
4. Add 1 mL of urea loading buffer 2× containing or not (*see* **Note 11**) tracking dyes to the resuspended pellet.
5. After washing out again the urea from the wells, load the sample into a large well and apply power (50 W).

3.3 RNA Recovery

1. After migration, i.e., when the bromophenol dye runs out, take down the gel and wrap it between two transparent plastic sheets. Place the wrapped gel on a silica plate and observe the RNA migration pattern by UV shadowing (*see* **Notes 12** and **13**).
2. Swiftly frame the band with an indelible felt pen to minimize the exposure time to UV radiations [12].
3. Using a clean scalpel blade, cut the RNA band of interest out of the gel.
4. Crush the gel band by pressing the acrylamide through a syringe with the plunger in a 50 mL BD Falcon™ conical tube.
5. To elute passively the RNA overnight, add 11 mL of water to the crushed gel and place the tube at 4 °C on a roller mixer.
6. Filter the sample through a 0.2 µm sterile filtration unit for 50 mL tubes (Nalgene®).
7. Add 10 % of ammonium acetate 3 M pH 5.2 to the filtrate and precipitate with 2.5 volumes of cold pure ethanol.
8. Spin the tubes containing the purified RNA at 27,000 × *g* for 20 min at 4 °C (*see* **Note 9**).
9. Discard the supernatant and resuspend the RNA pellet in a volume of water to obtain an RNA concentration around 20 mg/mL (*see* **Note 14**).
10. Determine the RNA concentration from OD measurements at $\lambda = 260$ nm.
11. Aliquot the sample and store the individual tubes containing the pure RNA at −80 °C.

4 Notes

1. Acrylamide and bisacrylamide powders are highly toxic chemicals. For this reason, ready-made solutions should be obtained from chemical providers. Avoid allowing acrylamide droplets from drying out on laboratory material used to prepare gel

solutions since acrylamide particles may be carried away in the airstream and hazardously be breathed by people.

2. To facilitate gel unmolding, glass plates can be siliconized on their side in contact with the gel.
3. The gel apparatus should possess a metal plate to homogenize the temperature within the gel during the separation run.
4. APS solutions should be as fresh as possible. Prepare stock solution on a 2-weekly basis and store at 4 °C.
5. The dissolution of urea in aqueous solutions is endothermic and thus requires heating. Use a glass beaker on a heated magnetic stirrer (~100 °C). Avoid overheat to prevent urea and acrylamide degradation.
6. Siliconizing fluids containing silane or siloxane derivatives are harmful and should be used with caution under well-ventilated hoods. Avoid skin and eye contact by wearing adequate individual protective equipment. This step is very useful in the case of thin gels with low acrylamide percentage (5–8 %), which is not the case in the protocol presented.
7. Since RNA is mostly single stranded under denaturing PAGE conditions, the choice of the best percentage of polyacrylamide can be guided by knowledge of the expected mobilities of xylene cyanol and bromophenol dyes (Table 7.7 of [1]).
8. Polymerized acrylamide is harmless and can be discarded in a regular trash can. Always neutralize acrylamide solution leftovers by polymerization before discarding.
9. Be aware of using a rotor dedicated to 50 mL conical bottomed tubes. Using a rotor for spherical bottomed tubes will result in destruction of the tube and loss of the sample and may cause imbalance, eventually damaging the centrifuge.
10. The presence of magnesium phosphate in the pellet makes it difficult to resuspend. Pipet up and down until the whole pellet is dissolved, which can take several minutes. The resulting solution will be cloudy.
11. To avoid co-migration of the RNA with tracking dyes, which could cause subsequent purification problems, mix the RNA sample with an equal volume of 8 M urea and load in the main wells, while a sample containing only loading buffer with tracking dyes is loaded in an independent well at the side of the gel.
12. When the RNA concentration is very high, the difference between the refractive index of the gel containing and not containing the RNA sample allows for naked-eye localization of the RNA bands during electrophoresis.
13. Use a handheld UV lamp with a 254/302 nm wavelength. Wear protective goggles, gloves, and lab coat since UV radiation is hazardous to the skin and eye.

14. According to the desired concentration, the volume of water may vary. Nonetheless apply the water directly onto the pellet, hold the tube steady until dissolution, or slightly agitate without dispersing the water drop. The volume can be adjusted after determining the OD of the sample.

References

1. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA
2. Easton LE, Shibata Y, Lukavsky PJ (2010) Rapid, nondenaturing RNA purification using weak anion-exchange fast performance liquid chromatography. RNA 16:647–653
3. Batey RT (2014) Advances in methods for native expression and purification of RNA for structural studies. Curr Opin Struct Biol 26:1–8
4. Chadalavada DM, Bevilacqua PC (2009) Analyzing RNA and DNA folding using temperature gradient gel electrophoresis (TGGE) with application to in vitro selections. Methods Enzymol 468:389–408
5. Henco K, Harders J, Wiese U, Riesner D (1994) Temperature gradient gel electrophoresis (TGGE) for the detection of polymorphic DNA and RNA. Methods Mol Biol 31:211–228
6. Milligan JF, Uhlenbeck OC (1990) Synthesis of small RNAs using T7 RNA polymerase. Methods Enzymol 180:51–62
7. Beckert B, Masquida B (2011) Synthesis of RNA by in vitro transcription. Methods Mol Biol 703:29–41
8. Meyer M, Nielsen H, Oliéric V, Roblin P, Johansen SD, Westhof E, Masquida B (2014) Speciation of a group I intron into a lariat capping ribozyme. Proc Natl Acad Sci U S A 111:7659–7664
9. Meyer M, Masquida B (2014) cis-Acting 5′ hammerhead ribozyme optimization for in vitro transcription of highly structured RNAs. Methods Mol Biol 1086:21–40
10. Treiber DK, Williamson JR (1999) Exposing the kinetic traps in RNA folding. Curr Opin Struct Biol 9:339–345
11. Wu M, Tinoco I (1998) RNA folding causes secondary structure rearrangement. Proc Natl Acad Sci U S A 95:11555–11560
12. Kladwang W, Hum J, Das R (2012) Ultraviolet shadowing of RNA can cause significant chemical damage in seconds. Sci Rep 2:517