Denaturing Polyacrylamide Gel Electrophoresis

Thin polyacrylamide gels that contain a high concentration of urea as a denaturant are capable of resolving short (<500 nucleotides) single-stranded fragments of DNA or RNA that differ in length by as little as one nucleotide. Such gels are uniquely suited for nucleic acid sequence analysis, which is required for all of the footprinting protocols in Chapter 6. Thicker gels are often used to purify oligonucleotides.

The following protocol describes the pouring, running, and processing of a typical "sequencing" gel which is 40-cm long with a uniform thickness of 0.4 mm, containing 7 M urea and 4% to 8% acrylamide. RNA samples are sometimes run on gels prepared with 8 M urea. A more detailed protocol with variations and extensive troubleshooting information is provided in *CPMB UNIT 7.6*. Some guidelines for pouring and running preparative gels are given in annotations to some steps.

POURING, RUNNING, AND PROCESSING DENATURING POLYACRYLAMIDE GELS

BASIC PROTOCOL

Materials

70% ethanol *or* isopropanol in squirt bottle
5% (v/v) dimethyldichlorosilane (Sigma) in $CHCl_3$
Denaturing acrylamide gel solution (see recipe)
TEMED
10% (w/v) ammonium persulfate (make fresh weekly and store at 4°C)
1× TBE electrophoresis buffer, pH 8.3 to 8.9 (*APPENDIX 2A*)
Samples for electrophoresis containing formamide and marker dyes

30 × 40–cm front and back gel plates
0.2- to 0.4-mm uniform-thickness spacers
Large book-binder clamps
60-mL syringe
0.2- to 0.4-mm shark's-tooth *or* preformed-well combs
Sequencing gel electrophoresis apparatus
Pasteur pipet *or* Beral thin stem (Beral Enterprises)
Power supply with leads
95°C heating block or water bath
46 × 57–cm gel blotting paper (e.g., Whatman 3MM)
Kodak XAR-5 X-ray film

NOTE: Many companies provide equipment needed for sequencing experiments; a list of suppliers is provided in *CPMB* Table 7.6.1.

Assemble the gel sandwiches

1. Meticulously wash front and back 30 × 40–cm gel plates with soap and water. Rinse well with deionized water and dry. Wet plates with 70% ethanol or isopropanol in a squirt bottle and wipe dry with Kimwipe or other lint-free paper towel.

   A typical preparative gel uses 20 × 16–cm plates.

2. Apply a film of 5% dimethyldichlorosilane in $CHCl_3$ to one side of each plate by wetting a Kimwipe with the solution and wiping carefully. After the film dries, wipe plate with 70% ethanol or isopropanol and dry with a Kimwipe. Check plates for dust and other particulates.

Contributed by Lisa M. Albright and Barton E. Slatko
Current Protocols in Nucleic Acid Chemistry (2000) A.3B.1-A.3B.5
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3. Assemble gel plates according to manufacturer's instructions, with the silanized surfaces facing inward. Use 0.2- to 0.4-mm uniform-thickness spacers and large book-binder clamps, making certain side and bottom spacers fit tightly together.

   Use thicker spacers (e.g., 1.6-mm) for preparative gels.

Prepare and pour the gel

4. Prepare 60 mL of desired denaturing acrylamide gel solution in a 100-mL beaker. (See Table A.3B.1 for appropriate acrylamide concentrations for resolving single-stranded DNAs. Consult Table A.3B.2 for preparative gels.) Thoroughly mix 60 μL TEMED, then 0.6 mL of 10% ammonium persulfate, into acrylamide solution immediately before pouring gel.

   To speed dissolution of urea, the gel mix can be heated before adding TEMED and ammonium persulfate; however, to prevent degradation of acrylamide, do not heat over 55°C. Allow to cool to room temperature (≤25°C) before adding the TEMED and ammonium persulfate to prevent polymerization while pouring the gel. If particulate matter remains, filter through a Whatman no. 1 filter paper in a funnel. To achieve slower polymerization, reduce amounts of TEMED and ammonium persulfate to 40 ì L and 0.4 mL, respectively.

5. Pour gel immediately. Gently pull acrylamide solution into a 60-mL syringe, avoiding bubbles. With short plate on top, raise upper edge of gel sandwich to 45° angle from the benchtop and slowly expel acrylamide between plates along one side. Adjust angle of plates so gel solution flows slowly down one side.

6. When solution reaches top of short plate, lower gel sandwich so that the top edge is ~5 cm above benchtop. Place an empty disposable pipet-tip rack or stopper underneath the sandwich to maintain the low angle. Insert flat side of a 0.2- to 0.4-mm shark's-tooth comb into the solution 2 to 3 mm below top of short plate, being very careful to avoid bubbles. Use book-binder clamps to pinch combs between plates so that no solidified gel forms between combs and plates. Layer extra acrylamide gel solution onto comb to ensure full coverage.

   Alternatively, insert teeth of preformed-well comb into gel solution and clamp as above.

   The comb should be the same thickness as the spacers.

   Rinse syringe with water to remove acrylamide.

Set up the electrophoresis apparatus

7. When gel polymerizes, remove bottom spacer or tape at bottom of gel sandwich. Remove extraneous polyacrylamide from around combs with razor blade. Clean spilled urea and acrylamide solution from outer plate surfaces with water. Remove shark's-tooth comb gently from gel sandwich without stretching or tearing top of gel. Clean comb with water so it will be ready to be reinserted in step 10.

Table A.3B.1 Migration of Oligodeoxynucleotides (Bases) in "Sequencing" Denaturing Polyacrylamide Gels Relative to Dye Markers

Polyacrylamide	Bromphenol blue	Xylene cyanol
5%	35 b	130 b
6%	26 b	106 b
8%	19 b	75 b
10%	12 b	55 b

If preformed-well comb was used, take care to prevent tearing of polyacrylamide wells. This comb will not be reinserted.

8. Fill bottom reservoir of gel apparatus with 1× TBE buffer so that gel plates will be submerged 2 to 3 cm in buffer. Place gel sandwich in electrophoresis apparatus and clamp plates to support.

   Sweep out any air bubbles at bottom of gel by squirting buffer between plates using syringe with a bent 20-G needle.

9. Pour 1× TBE buffer into top reservoir to ~3 cm above top of gel. Rinse top of gel with 1× TBE buffer using a Pasteur pipet or Beral thin stem.

10. Reinsert teeth of cleaned shark's-tooth comb into gel sandwich with points just barely sticking into gel. Using a Pasteur pipet or Beral thin stem, rinse wells thoroughly with 1× TBE buffer to remove stray fragments of polyacrylamide.

    If a preformed-well comb is used, this step is omitted.

11. Preheat gel ~30 min by setting power supply to 45 V/cm, 1700 V, 70 W constant power.

    Preparative gels are usually preheated and run at 20 to 40 V/cm, constant voltage.

Load and run the gel

12. Rinse wells with 1× TBE buffer just prior to loading gels, to remove urea that has leached into them.

13. Heat samples 2 min at 95°C in covered microcentrifuge tubes, then place on ice. Load 2 to 3 µL sample per well. Rinse sequencing pipet tip twice in lower reservoir after dispensing from each reaction tube.

14. Run gels at 45 to 70 W constant power. Maintain a gel temperature of ~65°C. Observe migration of marker dyes (Table A.3B.2) to determine length of electrophoresis.

    Temperatures >65°C can result in cracked plates or smeared bands; too low a temperature can lead to incomplete denaturation. To ensure even conduction of the heat generated during electrophoresis, an aluminum plate (0.4 cm thick, 34 × 22–cm) can be clamped onto the front glass plate with the same book-binder clamps used to hold the gel sandwich to the apparatus. The aluminum plate must be positioned so that it does not touch any buffer during electrophoresis.

Table A.3B.2 Concentrations of Acrylamide Giving Optimum Resolution for Purification of DNA Fragments Using Denaturing PAGE[a]

Acrylamide (%)	Fragment sizes separated (bases)	Migration of bromphenol blue (bases)	Migration of xylene cyanol (bases)
30	2-8	6	20
20	8-25	8	28
10	25-35	12	55
8	35-45	19	75
6	45-70	26	105
5	70-300	35	130
4	100-500	~50	~230

[a]Data, from Maniatis et al. (1975), are for single-stranded DNA; RNA will migrate slightly more slowly than DNA of the same sequence and length. Taken from *CPMB UNIT 2.12*.

Preparative gels are usually run at 20 to 40 V/cm, constant voltage. Turn off power when the position of the tracking dye indicates that the oligonucleotide has migrated sufficiently for isolation. Proceed with isolation method of choice.

Process and dry the gel

15. Fill dry-ice traps attached to gel dryer (if required) and preheat dryer to 80°C.

16. After electrophoresis is complete, drain buffer from upper and lower reservoirs of apparatus and discard liquid as radioactive waste.

17. Remove gel sandwich from apparatus and place under cold running tap water until surfaces of both glass plates are cool. Lay sandwich flat on paper towels with short plate up. Remove excess liquid and remaining clamps or tape. Remove one side spacer and insert long metal spatula between glass plates where spacer had been. Pry plates apart by gently rocking spatula.

    The gel should stick to the bottom plate. If it sticks to the top plate, flip sandwich over. Slowly lift top plate from the side with inserted spatula, gradually increasing the angle until the top plate is completely separated from gel.

18. Once plates are separated, remove second side spacer and any extraneous bits of polyacrylamide around gel.

19. Hold two pieces of dry 46 × 57–cm blotting paper together as one piece. Beginning at one end of gel and working slowly towards the other, lay paper on top of gel. Take care to prevent air bubbles from forming between paper and gel.

20. Peel blotting paper up; gel should come off plate with it. Gradually curl paper and gel away from plate as it is being pulled away.

21. Place paper and gel on preheated gel dryer. Cover with plastic wrap. Remove any bubbles between plastic wrap and gel by gently rubbing covered surface of gel from middle toward edges with a Kimwipe. Dry gel thoroughly 20 min to 1 hr at 80°C.

    When gel is completely dry, the plastic will easily peel off without sticking.

22. Remove plastic wrap and place dried gel in X-ray cassette with Kodak XAR-5 film in direct contact with gel. Autoradiograph at room temperature. After sufficient exposure time (usually overnight), remove X-ray film and process.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* **SUPPLIERS APPENDIX**.

Denaturing acrylamide gel solution

Reagent	Acrylamide concentration		
	4%	6%	8%
Urea (ultrapure; g)[a]	25.2	25.2	25.2
38% acrylamide/2% bisacrylamide (mL)	6.0	9.0	12.0
10× TBE buffer (*APPENDIX 2A*; mL)	6.0	6.0	6.0
H_2O (mL)	27	24	21
Total volume (mL)	*60*	*60*	*60*

[a]7 M final concentration.

Filter solution through Whatman no. 1 filter paper. Store 2 to 4 weeks at 4 °C.

CAUTION: *Acrylamide and bisacrylamide are hazardous.*

Solutions of acrylamide deteriorate quickly, especially when exposed to light or left at room temperature.

RNA samples are often run on sequencing gels containing 8 M rather than 7 M urea. In this case, use 28.8 g urea for 60 mL of gel solution.

LITERATURE CITED

Maniatis, T., Jeffrey, A., and deSande, H.U. 1985. Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. *Biochemistry* 14:3787-3794.

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