

2-D Polyacrylamide Gel Electrophoresis

This method was successful in our lab using prostate tissue and for our specific objectives. Investigators must be aware that they will need to tailor the following protocol for their own research objectives and tissue under study.

Solutions

TIP: Use electrophoresis grade reagents to prepare the following solutions:

A: 50 ml IEF Lysis Buffer

1. Add 21 g urea to 35 ml HPLC-grade H₂O to a 50 ml Falcon tube (final concentration 7 M).
2. Vortex vigorously for several minutes.
3. Add:
 - 7.6 g Thiourea
 - 2 g Chaps
 - 0.5 g Mega 10
 - 0.5 g OBG
 - 250 µl Triton X-100
 - 0.25 g Tris
 - 0.4 g DTT
 - 500 µl Pharmalytes or IPG buffer pH 3-10 (Amersham)
 - 500 µl β-mercaptoethanol
4. Add 10 µl tributylphosphine 2 mM, **under the hood** (2 mM final conc.)
5. Add Bromophenol Blue as indicator.
6. Check volume is 50 ml.
7. Vortex until all is dissolved (or attach tube to a rotator).
8. Aliquot 1 ml into microfuge tubes.
9. Store at -20°C.

B: 10X Electrophoresis Running Buffer (10 L) 0.25 M Tris, 1.92 M glycine, 1M SDS

1. Add 300 g Tris-base, 1441 g glycine, and 10 g SDS to ~7 L HPLC-grade H₂O.
2. Mix gently until dissolved.
3. Bring volume to 10 liters.

C: 30% Acrylamide Stock (1 liter)

1. Add 292 g acrylamide and 8 g piperazine diacrylamide (PDA) to 700 ml HPLC-grade H₂O, **under the hood**.
2. Stir to dissolve.
3. Bring volume to 1 L.
4. Filter through 0.45 µm pore size filter.
5. Store at 4°C in the dark.

D: Separating Acrylamide Gel

Below are the solution volumes required to prepare one 9-18% gradient gel. Prepare sufficient volume for the number of gels to be run.

Solution	Volume Units	9% gel	18% gel
1.5 M Tris-HCl, pH 8.8	ml	11.5	11.5
20% SDS	ml	0.23	0.23
30% Acrylamide	ml	14	28
TEMED	µl	11.7	11.7
10% APS	µl	117	117
HPLC-grade H ₂ O	ml	20	6
Total	ml	45.8	45.8

E: 50 ml Equilibration Buffer I

1. Mix together 18 g urea and 10 ml of 0.5 M Tris-HCl, pH 6.9.
2. Vortex vigorously.
3. Add 10 ml of 20% SDS and 200 mg DTT.
4. Invert gently several times.

5. Add 15 ml glycerol.
6. Vortex vigorously (or attach tube to a rotator for 10-15 min).
7. Add Bromophenol Blue as indicator.

F: 50 ml Equilibration Buffer II

1. Same as Equilibration Buffer I **EXCEPT** add 5.0 g Iodoacetamide **INSTEAD** of DTT.

G: Transfer Buffer

1. For 1 L:
25 mM Tris-HCl (3 g)
190 mM glycine (14.44 g)
20% methanol (200 ml)

Day One: Sample Preparation

A. Tissue Processing (See [Limitations of 2D-PAGE Electrophoresis](#) for number of cells needed)

- To lyse a 5-8 μm tissue section obtained from a paraffin-embedded block:
 1. Place the tissue section in a 1.5 ml Eppendorf tube.
 2. Add xylenes to cover the tissue.
 3. Vortex vigorously for ~15 sec.
 4. Incubate at RT for 5 min.
 5. Vortex again.
 6. Spin down for 3 min to pellet the tissue.
 7. Remove xylenes.
 8. Add 1 ml xylenes.
 9. Vortex 5-10 sec.
 10. Spin down.
 11. Remove xylenes.
 12. Speed vacuum the sample for a few minutes to evaporate the remaining xylene.
 13. Add 400 μl IEF buffer.
 14. Vortex vigorously for 1 min.
 15. Incubate for 5 min at RT.

16. Vortex vigorously for 1 min.
 17. Spin sample down at 14,000 g for 5-10 min at room temp.
- To lyse a 5-8 μm tissue section obtained from a polyester-embedded block:
Follow 1-15 above for a paraffin-embedded block section, **EXCEPT** use 100% EtOH instead of xylenes.
 - To a 5-8 μm tissue section obtained from a frozen block **OR** a microdissected tissue sample:
 1. Place the tissue section or microdissected tissue sample in a 1.5 ml Eppendorf tube.
 2. Add 400 μl IEF buffer.
 3. Vortex vigorously for 1 min.
 4. Incubate for 5 min. at RT.
 5. Vortex vigorously for 1 min.
 6. Spin sample down at 14,000 g for 5-10 min at room temp.
 - To lyse a paraffin-embedded section on a slide:
 1. Deparaffinize the tissue by immersing the slide into xylenes, twice for 5 min each.
 2. Allow the tissue section to dry.
 3. Add 200 μl of the IEF buffer to the tissue and pipet up and down several times.
 4. Remove the buffer into a microfuge tube.
 5. Scrape the tissue with a razor blade and transfer into the same microfuge tube.
 6. Add 200 μl to the lysates for a total of 400 μl .
 7. Vortex vigorously for 1 min.
 8. Incubate for 5 min at RT.
 9. Vortex vigorously for 1 min.
 10. Spin sample down at 14,000 g for 5-10 min at room temp.
 - To lyse a polyester-embedded section on a slide:
 1. Remove the polyester by immersing the slide into ethanol, twice for 5 min each.

2. Repeat steps 2-10, directly above, as for a paraffin-embedded section on a slide.
- To lyse a frozen section on a slide:
 1. Thaw the slide to room temperature.
 2. Immerse the tissue section into xylenes for 1 min.
 3. Repeat steps 2-10, directly above, as for a paraffin-embedded section on a slide.

B. Reswelling

1. Remove Immobiline Drystrips (Amersham Pharmacia Biotech) from -20°C and allow to equilibrate at RT.

TIP: The pH range of the strip used should be the same as the pH range of Pharmalytes or IPG buffer used in the IEF lysis buffer.
2. Notch the basic end of the strip to mark each sample.
3. Load the first sample into Reswelling trays (Immobiline DryStrip Reswelling Tray/Pharmacia #18-1004-31.)
4. Place DryStrips gel-side down into each slot.
5. Remove air bubbles by pressing down with a pipette tip.
6. Overlay completely with DryStrip Cover fluid (Amersham Pharmacia, #17-1335-01).
7. Repeat for every sample including the MW standard (2-D SDS-PAGE standards, pH range 4.5-8.5, MW 17,500-76,000, Bio-Rad, # 161-0320).
8. If samples are concentrated in one region of the strip, redistribute by pipetting.
9. Cover the tray with the lid.
10. Incubate overnight at RT to allow the strips absorb the samples.

Day Two: 1st Dimension

1. Clean the electrophoresis chamber (Pharmacia LKB Multiphor II) and the Immobiline strip tray and wipe out with paper towels and Kimwipes to remove mineral oil.
2. Place the tray on top of 50 ml DryStrip Cover fluid.
3. Remove strips.
4. Place on Whatman paper gel-side up.

TIP: Placing the strips gel-side down might result in protein loss and gel damage.

5. Leave for ~1 min.
6. Place into the electrophoresis chamber gel-side up.
7. Arrange the strips so that their edges are in one line.

TIP: Time is important to prevent crystallization.
8. Wet the pre-made IEF electrode strips (Amersham Pharmacia, #18-1004-40) with HPLC-grade H₂O.
9. Dry slightly between two pieces of Whatman paper.
10. Place 2 buffer strips on both edges of the strips and perpendicular to them, covering the top of the bromophenol blue on each side.
11. Make sure that the square end of each strip is at the cathode (Black/-) end and the pointed end is at the anode (Red/+) end and also that the anode and cathode electrode ridges are in the correct orientation.
12. Overlay liberally with DryStrip Cover fluid between the immobilon strips and outside the electrodes.
13. Electrophorese for 36-48 hrs, using the following sequence of settings:

	Voltage	Amps	Wattage	Time
1	500 V	100 mA	33 W	0.05 hrs
2	500 V	110 mA	70 W	1 hr
3	3500 V	141 mA	32 W	5 hrs
4	3500 V	70 mA	38 W	Until stopped

14. The bromophenol blue should be seen migrating towards the anode within at least 1 hr. By next day, the strips should be colorless.
15. If by the next day the bromophenol blue has not disappeared, running can be paused and the electrode strips can be replaced from whichever side. Continue running until the dye has disappeared.

Day Three: 2nd Dimension

A: Prepare Apparatus

1. Wash and scrub plates very well in soap and hot water.
2. Rinse in diH₂O.
3. Leave the plates to air dry or wipe with methanol-soaked Kimwipes.

4. Order plates in Protean-II Multi-Gel casting Chamber (Bio-Rad, #165-2025) as follows:
 - Bottom of chamber
 - Small plate, 20 cm
 - Spacers, 20 cm x 1 mm
 - Eared plates, 20 cm
 - Spacers, 20 cm x 1 mm
 - Large plate, 20 cm
 - Meylar sheet
 - Repeat as needed.
5. Fill the chamber with acrylic blocks.
6. Tighten the screws.
7. Tape the edges of the chamber to prevent leakage.

B: Prepare Gradient Acrylamide Gel (9-18%)

1. Add 9% gel solution to the center compartment of the distributor (mixing chamber) and 18% gel to the peripheral compartment (reservoir chamber).
2. Start the magnetic stirrer in the mixing chamber.
3. Remove air bubbles from the tubing by opening the valve slowly.
4. Allow the tubing to fill with gel solution, then close the valve.
5. Turn on the stirrer.

TIP: The stir bar should be between the openings between the mixing and reservoir compartments, but not on top of them.
6. Open the valve between the mixing and the reservoir chambers (upward).
7. Make sure the 18% solution is flowing into the mixing compartment.
8. Open the valve to start the flow of the acrylamide solution into the Protean chamber.
9. Allow reasonable flow. Fast flow results in loss of a gradient, whereas a slow flow results in polymerization of the solution in the tubing. In addition, the rate of flow changes with time due to the change of pressure. However, the chamber should be filled in at least 10 minutes.
10. Stop when the gel has reached 0.5 cm from the top of the glass plates.
11. Overlay the gels carefully with HPLC-grade H₂O using a syringe.
12. Cover with Saran Wrap.
13. Allow to polymerize overnight.

Day Four: 2nd Dimension (cont.)

1. Remove strips.
2. Place on Whatman paper, gel-side up, for 1 min.
3. Equilibrate in Equilibration Buffer I for 10-15 min.
4. Set-up gels in electrophoresis chamber (Protean II Multi-cell, Bio-Rad).
5. Make sure there is no leakage.
6. Equilibrate strips in Equilibration Buffer II.
7. Remove strips.
8. Place on Whatman paper one by one, gel-side up.
9. Identify notches.
10. Cut ~one inch from both sides.
11. Place gels with basic side closer to the anode (Red/+) and the acidic side closer to the cathode (Black/-) to be consistent.
12. Run gels at 40 mA/gel for 15 min or until dye front is about an inch into the gel.
13. Turn down voltage and run at 65-70 V constant overnight. (If in a hurry, set power supply at 360 mA and 500 V and run for 8 hrs).

Day Five: Transfer and Sequencing of Proteins

A: Transfer onto PVDF or Nitrocellulose Membranes

1. Soak gel in transfer buffer for 5-10 min.
2. Soak pre-cut transfer membrane and Whatman paper in transfer buffer.
3. Wet the transfer apparatus with transfer buffer.
4. Place 3-4 Whatman papers on bottom of the transfer apparatus.
5. Place membrane on top and remove air bubbles.
6. Place gel on top of the membrane and remove air bubbles.
7. Place 3-4 Whatman papers on top of the gel and remove any air bubbles.
8. Transfer for 3 hrs at no more than 25V (1-1.5 mA per cm² of the gel/membrane). Transfer for 5 hrs if more than 5-6 gels.

B: Gel Staining

See [Staining Protocols](#)

C: Excision of Protein Spots (for sequencing by MS)

TIP: Work under the hood to prevent the gel from being exposed to too much air, thus avoiding contamination with keratins

1. Xerox the gel and assign the spot(s) to be sequenced.
2. Cut out the protein spot for sequencing with a pipette tip.
3. Remove to a microfuge tube.
4. Chop up the gel piece with pipette tip.
5. Add a solution of 50% methanol/10% acetic acid to the gel pieces.
6. Incubate for 30 min.
7. Spin down.
8. Remove the supernatant.
9. The sample is ready for Mass Spectrometry Sequencing.