# Protocol

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## Two-Dimensional Gel Electrophoresis

### Introduction

Two-dimensional (2-D) electrophoresis is a powerful technique often used in proteomics research. This technique combines two high-resolution electrophoretic procedures that allows the characterization of proteins in a complex mixture. In the first-dimension gel (isoelectric focusing gel), solubilized proteins are separated according to their charge (isoelectric point, pI) on an acrylamide gel that has a pH gradient. Then, in the second dimension gel, these same proteins are separated according to their molecular weight through the SDS-PAGE method. Thus, the resulting 2-D gel contains thousands of spots representing separated proteins. This method is especially helpful when trying to separate proteins that are very similar except for very small changes, such as recombinant proteins. This protocol outlines an entire procedure for the generation of a two dimensional gel starting from a one dimensional gel.

### Materials

For First-Dimension Gels

- Urea (GoldBio Catalog # U-200)
- 30% acrylamide/1.8% bisacrylamide *(see protocol)*
- Ampholytes, pH between 4 and 8
- NP-40 or other similar nonionic, non-denaturing detergent
- TEMED (N,N,N',N' tetramethylethylenediamine)
- 10% ammonium persulfate *(see protocol)*
- 0.085% phosphoric acid *(see protocol)*
- 0.02M NaOH *(see protocol)*
- Protein samples
- Concentrated bromophenol blue (GoldBio catalog # B-092) *(see protocol)*
- Alconox cleaning solution
- 1.0 to 3.0 mm inner diameter glass gel tubes (~1.5 in. longer than the width of the second-dimension gel; 4 to 6 mm outer diameter)
- 2.5 to 3.0 cm inner diameter gel casting glass tube (~1 cm shorter than gel tubes small vacuum flask)
- Small vacuum flask
- 50 µl, 1 ml and 20 ml syringes
- 0.2 or 0.45 µm filter capsule
- Single edge razor blade
- Rubber grommets
- Tube cell
- 22 G hypodermic needle (2 in long)
- 200 µl pipette tip
- 1 dram gel vials

For Second-Dimension Gels

- 30% acrylamide/0.8% bisacrylamide *(see protocol)*
- Gel buffer *(see protocol)*
- 10% (w/v) SDS *(see protocol)*
- TEMED
- 10% ammonium persulfate
- Isobutyl alcohol, $H_2O$-saturated

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- Stacking gel buffer (optional)
- Completed First-dimension gel
- Equilibration buffer *(see protocol)*
- Hot 0.5% and 1% agarose (keep in boiling water bath) *(see protocol)*
- Protein molecular weight standards
- SDS solubilization buffer *(see protocol)*
- Reservoir buffer, pre-chilled to 10° to 20°C *(see protocol)*
- Coolant (running tap water or circulating refrigerated water bath)
- Gel plates, one long and one short
- 1.5 mm spacers (~14 cm × 14 cm × 14 cm)
- Casting stand
- Gel identification tag
- Nylon screen
- 5 × 15 cm glass plate
- Electrophoresis cell

For Acrylamide/bisacrylamide solutions
First Dimension

- 30 g Acrylamide
- 1.8 g Bisacrylamide (GoldBio Catalog # B-900)
- $H_2O$ to 100 ml

Second Dimension

- 300 g Acrylamide
- 8.0 g Bisacrylamide
- $H_2O$ to 1 liter
- Filter through 0.2 to 0.45 µm filter. Store in tightly capped amber bottles at 4°C. Discard after 30 days, as acrylamide gradually hydrolyzes to acrylic acid and ammonia.

**CAUTION: Acrylamide monomer is neurotoxic. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth. Wear a mask when weighing out the solid acrylamide.**

For Agarose Solution (0.5% and 1%)

- 0.25 g Agarose (GoldBio Catalog # A-201) for 0.5% solution, 0.5 g agarose for 1% solution.
- 50 ml Reservoir buffer
- Heat in a boiling water bath to dissolve the agarose and keep in a boiling water bath. Prepare fresh each time.

For Ammonium Persulfate, 10%

- 10 g Ammonium persulfate
- $H_2O$ to 100 ml
- Store refrigerated for ≤ 2 weeks.

For Bromophenol blue stock solution, 1%

- 100 mg Bromphenol blue
- 60 mg Tris base (50mM final)
- Double-distilled $H_2O$ to 10 ml
- Prepare fresh.

For Alconox Cleaning Solution

- Follow instructions on package.

For concentrated bromphenol blue

- 50% Aqueous glycerol (v/v)
- 0.01 mg/ml Bromphenol blue
- Prepare fresh.

For equilibration buffer

- 3.75 g Tris base (GoldBio Catalog # T-400)
- 25 ml Glycerol
- 5.25 g SDS
- 333 mg Dithiothreitol (DTT) (GoldBio Catalog # DTT)
- Dissolve Tris base in $H_2O$ and adjust to pH 6.8 with 6M HCl. Add other ingredients. Add $H_2O$ to 250 ml final volume. This buffer can be stored for up to 1 week at room temperature.

For gel buffer

- Dissolve 90.8 g Tris base in 300 ml $H_2O$. Adjust to pH 8.6 with 6M HCl. Add $H_2O$ to 500 ml. This buffer can be stored for several weeks at 4°C.

For 0.02M NaOH

- Just before using, add 0.5 ml of 10M NaOH to 250 ml freshly deaerated water. Store up to 1 month at room temperature.

**Note: It is especially important to make 0.02M NaOH with deaerated water.**

For phosphoric acid, 0.085%

- Just before using, dilute 300 ml of 0.85% phosphoric acid to 3.0 liters with deaerated water. Store up to 1 month at room temperature.

For reservoir buffer

- 15.0 g Tris base
- 72.0 g Glycine (GoldBio Catalog # G-630)

- 5.0 g SDS
- $H_2O$ to 5 L
- For convenience, make up as a 10x stock or store the pre-weighed dry ingredients in packets for future use. The 10x stock can be stored for several weeks in the refrigerator.

For SDS solubilization buffer

- 0.1 g 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) (GoldBio Catalog # C-870)
- 0.2 g SDS
- 0.1 g DTT
- 1.0 ml Glycerol
- $H_2O$ to 10 ml total volume
- Store aliquots at -70°C.

For Stacking gel buffer

- 15.0 g Tris base
- 1.0 g SDS
- Dissolve Tris base and SDS in 200 ml $H_2O$. Adjust to pH 6.8 with 6M HCl. Add $H_2O$ to 250 ml.
- The 5x stock can be stored for several weeks in the refrigerator.

## Method

Prepare and pour the gels

1. Mark clean, dry 1.0- to 3.0 mm-i.d. (inside diameter) gel tubes to indicate the desired height of the gel (usually the same as the width of the second-dimension gel).

   **Note: The most commonly used type of isoelectric focusing apparatus is the cylindrical unit. Gel tubes are available with a wide range of inside diameters: however, for the highest resolution, tubes of 1.5 mm-i.d. are preferred. Tubes up to 3 mm-i.d. may be used if it is necessary to load large sample volumes or larger amounts of protein.**

2. Place a rubber band around the gel tubes so that they form a tight bundle (~12 tubes fit into a bundle).

3. Hold the bundle vertically on a flat surface and push down on the tops of the tubes so that the bottoms are even.

4. Carefully seal one end of the 2.5- to 3.0 cm-i.d. gel-casting glass tube with three or four layers of Parafilm to form a strong, water-tight seal.

5. Place the bundle of gel tubes inside the gel casting tube and support the glass tube in a vertical position with a ring stand clamp to allow the sealed end of the glass tube to rest on a solid surface.

6. Add 8.25 g urea, 5.0 ml water, 2.0 ml of 30% acrylamide/1.8% bisacrylamide and 0.75 ml ampholytes, pH between 4 and 8, to a small vacuum flask. Add a small stir bar to the flask. Place the flask in a warm water bath on a magnetic stirrer and stir just until the urea is in solution; do not heat the solution to > 30°C.

   **Note: This makes 15 ml acrylamide solution, sufficient for twenty 1.5 × 160 mm gels.**

7. Deaerate the solution by applying a strong vacuum for 2-3 minutes.

8. Add 0.3 ml of NP-40 or similar type of detergent.

   **Note: Do not add the NP-40 before deaeration because it will foam.**

9. Pour solution into a 20 ml syringe fitted with a 0.2 or 0.45 µm filter capsule and force through the filter.

10. Add 10 ml TEMED and swirl. Add 70 ml of 10% ammonium persulfate and swirl. Immediately pipet the gel solution into the space between the gel tubes and the large glass tube.

    **Note: Mix well to ensure even polymerization. Work quickly, the gel solution will begin to polymerize in ~3 minutes.**

11. Gently run water down the outside of the gel tubes using a wash bottle. Add water until the level of the acrylamide solution inside the tubes reaches the desired height.

    **Note: As water is layered on top of the gel solution it will force the solution up the tubes from the bottom. There is no need to overlay the gel solution inside the tubes. Allow ≥ 1 hour for polymerization.**

Set up the gels in the tube cell

1. Remove the Parafilm from the bottom of the gel casting tube and push the gel tubes containing the polymerized gel out the bottom. Cut across the gel-tube bottoms to remove excess acrylamide with a single-edge razor blade. Rinse the bottom of the gel tubes under running deionized water to remove residual acrylamide.

2. Place rubber grommet on the top of each tube, making sure that the top surface of the gel is visible below the grommet; ~5 mm of the gel tube should be visible above the grommet.

3. Seat the tube and grommet assemblies in the holes of the upper buffer reservoir of the tube cell. Plug any unused holes with rubber stoppers.

4. Fill the lower reservoir with ~3 L of 0.085% phosphoric acid.

5. Place the upper reservoir into lower reservoir and adjust lower buffer level if needed.

**Note: The buffer should cover the entire gel for good heat dissipation.**

6. Fill the upper buffer reservoir with 250 ml of 0.02M NaOH.

7. Fill the gel tubes to the top with 0.02M NaOH using a 1 ml syringe equipped with a 22 G hypodermic needle. Be careful to eliminate any air bubbles in the gel tubes.

8. Connect the tube cell to the power supply. The black (-) lead goes to the upper reservoir. Prefocus the gel for 1 hour at 200 V constant voltage. Disconnect the tube cell from the power supply.

Load the samples and run the gels

1. Layer protein samples on top of the gels through the upper buffer with a 50 µl syringe.

**Note: The maximum amount of total protein that can be loaded onto a first-dimension gel varies depending upon the nature of the sample. Samples such as whole-cell lysates which contain a large number of proteins of widely varied isoelectric point (pI) can contain much more total protein than a sample of a single highly purified protein. As much as 100 to 150 µg of a protein mixture can be loaded on a first-dimension gel 1.5 mm in diameter, while a tenth that amount of highly purified protein would probably be an overload. For gels 1.5 mm in diameter, 10 to 20 µl of sample is preferred; however, up to 30 µl can be applied. Alternatively, place the samples directly on the gel, then overlay with 10 to 20 µl half-strength solubilization buffer (diluted 1:2 with water). This will protect samples from exposure to the basic upper buffer. Fill the remainder of the tube with 0.02M NaOH to eliminate any bubbles.**

2. Place the lid on the upper reservoir and attach the electrical leads to a power supply.

3. Turn on the power supply and adjust to the desired settings at constant voltage.

**Note: For 1.5 mm-i.d., 16 cm long gels, 700 to 800 V (constant voltage) for 16 hours (11,000 to 13,000 V/hr) works well for most samples.**

4. Reduce the voltage setting to zero and turn off the power supply to end the run. Add ~1 µl concentrated bromophenol blue to the top of each gel with a 50 µl syringe.

> **Note: The bromophenol blue will quickly diffuse into the gel. At the end of the run, the gels may be noticeably shorter and the bromophenol blue may stop short of the top of the gel. In this case, the power can be applied for a few minutes and the dye will migrate into the top of the gels. The blue dye will mark the basic end of the first-dimension gel and serve as the tracking dye in the second-dimension separation.**

Extrude the gels

1. Extrude the gels from the tubes using water pressure from a 1-ml syringe fitted with a 200 µl pipette tip (cut off ~1 cm of the large end of the tip so it fits on the syringe).

> **Note: Gels with a diameter > 2.0 mm may have to be loosened by inserting a blunt 26 G, 9 cm long needle between the gel and the wall of the tube while injecting water. Rotate the tubes so the needle will pass over the entire circumference of the gel while continuously injecting water. Repeat this operation at the opposite end of the tube and the gel should slide out easily.**

2. Place each gel in a labeled gel vial. The gels can be stored at -70°C for many weeks or used immediately.

   Soak gel tubes overnight in Alconox cleaning solution, then rinse thoroughly under running deionized tap water for 15 minutes. Remove excess water from the gel tubes with suction and allow them to dry.

For second dimensional gels – Prepare and pour the gel

1. Assemble the gel plates by placing 1.5 mm spacers vertically between a long and short gel plate. The side where the long plate protrudes is the top.

> **Note: Gel thickness is usually equal to the diameter of the isoelectric focusing gels, but this is not absolutely necessary.**

2. Position clamps on each side of the gel sandwich over the spacers and place on the casting stand. Be sure the plates and spacers are properly aligned, then tighten the clamps and cams to get leak-proof seal. Make adjustments so that plates are level and vertical.

3. Place the gel identification tag between the glass plates so that it rests in the lower right hand corner.

4. Prepare the gel solution by combining 30% acrylamide/0.8% bisacrylamide, gel buffer and water in a vacuum flask.

5. Deaerate the solution by applying vacuum for 5 minutes.

6. Add 10% SDS and TEMED and swirl, then add 10% ammonium persulfate and swirl.

7. Fill the gel sandwich to 5 mm below the top of the short plate and overlay with $H_2O$ saturated isobutyl alcohol or water.

> **Note: A stacking gel is not usually required when running second-dimension gels. If a stacker is desired, stop the gel 1.5 cm from the top of the short plate, overlay with $H_2O$-saturated isobutyl alcohol, and allow to polymerize for 1 hour. Remove the overlay and fill the remaining space with stacking gel solution made by combining 4.8 ml stacking gel buffer, 3.8 ml water and 1.4 ml 30% acrylamide/0.8% bisacrylamide (all deaerated) with 200 µL of 10% ammonium persulfate and 8 µl TEMED. Pipet onto the separating gel, overlay with $H_2O$-saturated isobutyl alcohol, and allow to polymerize for 30 minutes.**

8. Allow the second-dimension gel to polymerize ≥ 1.5 hour.

Loading first-dimension gel onto second-dimension gel

1. If first-dimension gel is frozen, thaw to room temperature. Add equilibration buffer to completely cover the gel.

> **Note: The time the gel is in equilibration buffer containing SDS can vary from seconds to several minutes depending upon the sample. Most proteins run well without the introduction of SDS from equilibration buffer; however, a few proteins do not. Leaving gels in equilibration buffer for > 10 to 15 minutes can result in loss of proteins.**

2. Pour the gel and equilibration buffer onto a nylon screen placed over a beaker.

3. Place the first-dimension gel on a 5 × 15 cm glass plate (Parafilm is not rigid enough). Using a spatula, lay the gel out straight along one edge of the glass plate.

4. Pipet a very thin layer of hot 0.5% agarose on the top of the slab gel to be loaded.

> **Note: This can easily be done by putting ~0.1 ml hot agarose on the upper left corner of a gel and quickly tilting the gel to the right so that the agarose will flow across the gel surface.**

5. Using the spatula, slide the first-dimension gel off the glass plate and place it across the top of the slab gel. Orient the first dimension gel with the blue (basic) end to your right.

**Note: Take care that no air bubbles get trapped between the first and second-dimension gels and that the first-dimension gel does not get stretched or compressed.**

6. Pipet a thin layer of hot 0.5% agarose over the first-dimension gel to seal it in place. Allow the agarose to solidify.

**Note: Protein molecular-weight markers may be run as one-dimensional separations on the sides of the second-dimension gel. Solubilize marker proteins in SDS solubilization buffer by boiling for 5 minutes, then dilute 1:1 with hot 1% agarose solution and draw up the hot solution in a glass tube the same diameter as the first-dimension gel. A short piece of the solidified agarose can be applied to one or both sides of the second-dimension gel and held in place with 0.5% agarose.**

Run and analyze the gel

1. Mount the gels on the electrophoresis cell, with the short plate going towards the center of the unit.

2. Fill the upper and lower reservoirs with pre-chilled reservoir buffer.

3. Attach tubing for coolant to the in and out ports and start the flow of coolant to maintain the temperature of the tank buffer at 10°C to 20°C during the run to ensure that the gels are adequately cooled.

**Note: The tank buffer should be the same temperature for each run.**

4. Attach the electrical leads to the power supply (upper reservoir connected to the negative lead). Electrophorese at 15 to 20 mA/gel until the tracking dye reaches the end of the gel (or 3 to 5 mA/gel overnight).

5. Reduce the voltage setting to zero and turn off the power supply at the end of the run. Remove the gels from the electrophoresis unit and take off the clamps. Pry the glass plates apart with a spatula.

6. Stain the gels, process the gels for immunoblotting or autoradiograph the cells.

## Associated Products

- Urea (GoldBio Catalog # U-200)
- Concentrated bromphenol blue (GoldBio catalog # B-092)
- Bisacrylamide (GoldBio Catalog # B-900)
- Agarose (GoldBio Catalog # A-201)
- Tris base (GoldBio Catalog # T-400)

- Dithiothreitol (DTT) (GoldBio Catalog # DTT)
- Glycine (GoldBio Catalog # G-630)
- 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) (GoldBio Catalog # C-870)

## References

Adams, L. D. & Gallagher, S. R. (2004). Two-dimensional gel electrophoresis. *Current Protocols in Molecular Biology*, 10-4.

O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell, 12*(4), 1133-1142. Doi:10.1016/0092-8674(77)90176-3.