

SDS-PAGE

Resolving gel

For large gel, thin thickness

10 mL 30% acrylamide
7.5 mL 4X soln* Tris pH 8.9
12.5 mL ddH₂O
Add 300µL 10% APS and
12 µL TEMED

For large gel, middle thickness

13.2 mL 30% acrylamide
10 mL 4X soln* Tris pH 8.9
16.8 mL ddH₂O
Add 400µL 10% APS and
16 µL TEMED

For mini gel (10%)

2.0 mL 30% acrylamide
1.5 mL 4X soln* Tris pH 8.9
2.5 mL ddH₂O
Add 60µL 10% APS and
2.4 µL TEMED

Stacking gel

large gel, thin thickness

2.6 mL 30% acrylamide
5 mL 4X soln* Tris pH 6.8
12.4 mL ddH₂O
Add 200µL 10% APS and
8 µL TEMED

large gel, middle thick

3.9 mL 30% acrylamide
7.5 mL 4X soln* Tris pH 6.8
18.6 mL ddH₂O
Add 300µL 10% APS and
12 µL TEMED

For mini gel (3.9%)

0.65 mL 30 % acrylamide
1.25 mL 4X soln* Tris pH 6.8
3.1 mL ddH₂O
Add 50µL 10% APS and
4 µL TEMED

For large gels, seal with 1 mL of resolving gel. Remove 2 mL and when sealant is polymerized, the remaining 1 mL of gel will be polymerized. Add to 2 mL of resolving gel, 4µL TEMED and 60µL 10% APS to polymerize sealant.

Pour the resolving gel

- 1. Clean glass plates with soap and water, then with ethanol. Assemble the glass plates and spacers.**
- 2. Seal the large gel plates by pouring 1 mL of resolving gel (after adding the APS/TEMED) into the plates and tilting them to spread the sealant. Allow most of excess sealant to run out, but save a small amount of excess to sit on the bottom to make sure it seals O.K. While this is going on, degas (for 10 minutes) the remaining quantity of unpolymerized resolving gel.
For the minigel, attach the glass plate assembly to the apparatus and place the whole gimisch in a small, flat tupperware container. With a pasteur pipette, seal the bottom of the plates by spreading 1% agarose in SDS PAGE electrophoresis buffer along the bottom of the glass plates so it goes up the crack by capillary action.**
- 3. Transfer the degased resolving gel to a beaker and add APS/TEMED. Mix. Quickly add the resolving gel solution to the center of the plates to a height about 4 cm from the top for the large plates. For the minigel, add the resolving gel with a pasteur pipette.**
- 4. Quickly add water-saturated isobutanol to the top of this until the level reaches the top of the plates. Isobutanol will prevent oxygen from getting into the gel which could oxidize it and inhibit polymerization.**
- 5. The resolving gel should polymerize in 30 minutes.**
- 6. Add the stacking gel reagents to a flask and degas for ten minutes.**

Pour the stacking gel

- 7. Pour off the isobutanol. Pour water several times into the gel plate space to rinse off all the isobutanol. Dry the watery interplate surface with a piece of Whatmann paper.**
- 8. To polymerize the stacking gel, add APS/TEMED, mix, then pour on top of the polymerized resolving gel.**

9. Insert the comb straight on down, then pour a little more stacking gel on the sides of the comb to fully seal it. Remove any bubbles from underneath the comb, if possible, by moving the comb gently from side to side so the bubbles get into the space in between and float up.

10. The stacking gel should polymerize in 20 to 30 minutes.

Load the gel

11. Attach the large gel plates containing the polymerized gel to the apparatus via the clips provided. (For the minigel, the plates are attached to the apparatus when the gel is poured.)

12. Pour Tris-glycine electrophoresis buffer into the upper and lower chambers. The large gel takes about 1 liter, the minigel takes about 200 mL.

13. Remove bubbles trapped at the bottom of the glass plates in the large gel with a syringe.

14. Flush the wells with a syringe just before loading to get rid of any unpolymerized polyacrylamide that may seep in.

15. When loading the gel, load something (1X loading buffer in blank lanes) in every lane and the dye front will migrate more evenly.

16. Run large thin gels at a constant current of 20 mA. After the dye front enters the resolving gel, you can turn the current up to 30 mA. Where you stop the gel will depend on how big the smallest protein is that you want to visualize. If you wait until the dye front just flows out of the gel, it should take about 2 1/2 - 3 hours for a large, thin gel to run. A thick gel could take 6 - 7 hours.

Run minigels at 15 mA. It should take about an hour to run.

Western blotting

Transfer the gel

1. While the gel is running, prepare the transfer buffer, and cut out a piece of nitrocellulose and four pieces of Whatmann filter paper for the transfer. Our Biorad tank takes about 3 liters of transfer buffer.
2. Cut out the Whatmann filter papers so they each are slightly larger than the gel in each dimension--about 1/4 to 1/2 cm larger. Cut out the nitrocellulose so that it is slightly smaller than the Whatmann filter papers but still covers the entire surface of the gel.
3. Float the nitrocellulose filter on the surface of a tray of deionized water and allow it to wet from beneath by capillary action. Then, submerge the filter in the water for five minutes to displace trapped air bubbles.
4. Remove the plates from the apparatus. Remove the spacers and pry off one of the plates by inserting a spatula and twisting the plate up. The gel should now be stuck to one of the plates.
5. Carefully float the gel off the plate into a tupperware container containing transfer buffer. Let it equilibrate in the transfer buffer for 15 minutes.
6. When the gel has equilibrated, construct a "sandwich" for the transfer as follows:
 - a. Place some transfer buffer in a large glass or tupperware container and place the plastic frame inside so the green side is lying in the buffer and the white side is folded out.
 - b. Place a sponge on top of the green side, then stack two sheets of the Whatmann paper you cut out on top. These things should be submerged in the buffer so that they become completely wet.
 - c. Place the nitrocellulose filter, labeled appropriately, on top, then carefully position the gel on top of this.
 - d. Add two more pieces of Whatmann filter paper, then a second sponge. Then press everything down into the buffer and squeeze out any air bubbles that might be trapped in the sandwich using a pipette as a roller.

e. Close the sandwich by folding the white side over onto the green and locking it in place. Then immerse the sandwich in our transfer tank to which transfer buffer has been added. Since the proteins will migrate toward the positive end, make sure that the nitrocellulose side is closest to the positive electrode. That is, the green side of the sandwich should be facing the red side of the tank.

7. Transfer the gel by running at 25 V overnight. Make sure to have the cooling unit on at 4 C so that cold water can circulate through the tank while the transfer is taking place. Otherwise, if the buffer gets hot, bubbles of air may come out of solution and become trapped in the sandwich.

Probing the blot with an antibody

1. Remove the blot from the transfer unit and block by placing in 5% milk blotting solution for 1 hour with shaking.

2. Incubate in primary antibody on our rocker for one hour. Typically, the primary antibody is diluted 1:1 or so if it is from the supernatant of a hybridoma cell line all the way up to 1: 1000 or more, if say it is purified from ascites fluid. We have been diluting primary in 5% milk blotting solution. Small volumes of 5 mL or less of diluted primary antibody can be added to a blot as large as 250 cm² if the plastic heat sealable bags we have are used. You can cut and seal the bag so the area around your blot is minimized. Add the primary, smooth out bubbles with a pipette, and seal.

3. Wash the blot three times with wash buffer, 5-10 minutes each time.

4. Incubate the blot in secondary antibody for one hour on a rocker. Most of our primary antibodies are from mice, so the goat antimouse antibody from the ECL kit can be used. The blot can be incubated in 10 mL of wash buffer that is 2% BSA and to which secondary antibody has been added at a 1:5000 dilution.

5. Wash the blot four times with wash buffer, 5-10 minutes each time. Between washes, rinse briefly with H_2O

6. Toward the end of the washes, prepare for exposing the blot to film. Take an old 8" X 10" film and place Saran wrap around one side. Then, take a second piece of Saran wrap and place it around

the same side such that there is an opening that the blot can be slid into.

7. When the blot is finished washing, blot the excess wash onto a Whatmann filter. Place the blot onto a piece of Saran wrap and add the chemiluminescence reagents. As little as 1 mL of each of the two reagents can be mixed in a tube and added to a 250² blot. Fold the Saran wrap over the blot and make sure that the small volume of reagents trapped in the space in between contacts the entire surface of the blot. Leave the reagents on for about one minute.

8. Blot off the excess, shove the blot into the case you constructed, and expose to film. A one minute exposure can be tried first as a test exposure. Then, other exposure times can be used depending on the intensity you see with this.

9. Blots can also be stripped and reprobed. Incubate in stripping solution for 30 minutes, then wash twice in washing solution for 10 minutes each time. You are now ready to block with milk blotto solution and proceed from the beginning as described above.

Stock Solutions

Milk blocking solution

<u>100 ml</u>	<u>200 ml</u>	<u>500 ml</u>	
5 g	10 g	25 g	dry milk
10 ml	20 ml	50 ml	1 M Tris pH 8.0
0.875 g	1.75 g	4.38 g	NaCl
50 μ l	100 μ l	250 μ l	0.05% TWEEN 20
20 mg	40 mg	100 mg	sodium azide

1 M Tris HCl pH 8.0

For 1000 ml: Dissolve 121.1 g Tris Base in 800 mL ~~dH~~ Adjust pH to 8.0 by adding HCl and bring up to volume with ~~dH~~

5X Wash buffer

1000 ml

400 ml 1 M Tris pH 8.0

45 g NaCl

5.0 ml TWEEN 20

Bring up to volume of 1000 ml with about 595 ml ~~dH~~

Tris-glycine electrophoresis buffer

1000 ml

25 mM Tris base

250 mM glycine pH 8.3

0.1% SDS

A 5X stock can be made by dissolving 15.1 g of Tris base and 94 g of glycine in 900 ml of deionized ~~dH~~. Then, 50 ml of a 10% (w/v) stock solution of electrophoresis-grade SDS is added, and the volume is adjusted to 1000 ml with ~~dH~~.

1X SDS gel-loading buffer

50 mM Tris-Cl (pH 6.8)

100 mM dithiothreitol

2% SDS

0.1% bromophenol blue

10% glycerol

1% SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should then be added, just before the buffer is used, from a 1 M stock.

Transfer buffer

3 liter

7.5 g boric acid

11.25 g sodium borate

in 3 liter in ddH₂O

1.5 liter

3.75 g boric acid

5.63 g sodium borate

in 1.5 liter ddH₂O

Stripping solution

200 ml

12.5 ml 1 M Tris HCl pH 6.8

40 ml 10% SDS

1.4 ml mercaptoethanol

Bring up to 200 ml volume with ddH₂O