

Western Blot

Equipment and Reagents:

- Biorad Mini-Protean 3 Cell system—Cat#165-3301
- Precision Plus Kaleidoscope molecular weight Standards—Biorad Cat#161-0375
- Laemmli sample Buffer (LSB)—Biorad cat#161-0737
- Tris-HCl 4-20% pre-cast linear gradient gel Biorad Cat#161-1123EDU
- Running Buffer (Tris/Glycine/SDS) Biorad—cat#161-0732
- β -Mercaptoethanol
- Tween-20—Sigma cat #P9416
- 10x Tris buffered saline – Biorad cat# 170-6435
- 10x Tris buffered saline(25mM)/glycine(192mM) —Biorad cat#161-0771
- StartingBlock (TBS) Blocking Buffer —Pierce Cat#:37542
- Electrode assembly and power source

1. Prepare protein samples
 - a. Choose protein sample with lowest concentration and determine how many ug of protein will be in 20uL (each well holds max 25uL)
 - b. Calculate the volume of each other protein sample to use such that you will be adding the same mass of protein from each sample
 - c. Add the calculated volumes of each sample to a new tube
 - d. Divide the sample volume by 5 and add that volume of LSB to the tube
 - e. Spin down contents of tubes, heat on block at 95C for 5 min, spin down again and hold on ice
2. Prepare 900mL SDS Running buffer by diluting 90mL of 10 \times Running Buffer (Tris/Glycine/SDS)
3. Prepare 1.5L transfer buffer (25mM Tris, 192mM Glycine) by diluting 150mL of 10 \times Tris/glycine and add 20% v/v methanol and move to 4 $^{\circ}$ C
 - a. Can prepare large stock of this and hold at 4C
4. Open gel packet (from 4C) and gently remove comb from the gel.
5. Remove bottom tape from the gel and place into the slots at the bottom of each side of the Electrode assembly. The short plate must face inwards towards the notches of the U-shaped gasket.
 - a. If only running one gel, use an empty glass plate system on the other side
6. Lift gel cassettes into place against the green gaskets and slide into the clamping frame
7. Press down on the Electrode Assembly while closing the cams of the Clamping frame to form the inner chamber to insure proper seal of the short plate to notch in gasket—this prevents leaking

8. Lower the inner chamber into the Minitank. Fill the inner chamber first, then outer chamber, with running buffer until the gel is submerged
9. Load 5uL of protein marker into the first and last lanes.
10. Load samples slowly, giving sample time to settle evenly in the bottom of the well. DO NOT puncture the bottom of the well with pipette tip.
11. Place lid on the Mini tank. Incorrect orientation is prevented by a stop. Align color coded banana plugs and jacks.
12. Insert the electrical leads into a power supply with proper polarity
13. Apply power to the Mini-PROTEAN 3 cell and begin electrophoresis; 200volts constant is recommended for SDS-PAGE. Run for 35min at room temp—until loading dye at bottom.
14. After electrophoresis is complete, turn off power supply and remove leads.
15. Remove lid and carefully lift out the inner chamber assembly. Pour off and discard the running buffer—ALWAYS pour off buffer before opening cams.

Blot Transfer and Detection

Reagents

- Mini transblot gel assembly
- Powdered milk
- Roller to squeeze out bubbles – we use a shortened serological pipet
- Magnetic stir bar and stirring plate
- Heat sink (e.g, plastic container filled with ice. Re-freeze after every use)
- Detection marker and developer – we use Supersignal West Pico Stable Peroxide solution, HRP conjugated to an antibody for the primary antibody's host (e.g, if your primary antibody was produced in rabbit, use anti-rabbit HRP). HRP conjugates come with developing reagents.

DO NOT handle membrane with bare hands; always wear gloves and use clean forceps.

1. Cut the membrane to the size of the gel if needed
2. Wet the membrane by placing it in transfer buffer and allowing it to moisten
3. Cut and completely saturate the filter paper (cat#170-3932) and filter pads with transfer buffer. Beware of trapping air bubbles.
 - a. Can set this up in order of layering (i.e. black side of gel holder → filter pad → filter paper in one dish; membrane in own dish; filter paper and pad in third dish)
4. Gently separate the two plates of the gel cassette using the lever in the gel box
5. Remove the gel gently with tweezers and place on filter paper with correct orientation

6. Rinse BioRad Protean Minitank with distilled, deionized water after use.
7. Open the gel holder and layer the pieces on the holder in the following fashion being careful not to trap bubbles and using forceps:
Black → Filter pad → Filter paper → Gel → Nitrocellulose membrane → Filter paper → Filter pad → Clear
8. After placing the second filter paper, roll with the roller to completely remove bubbles.
9. Close the cassette. Hold it firmly so that the sandwich will not move and secure the latch. At this point movement of the sandwich may alter gel membrane contact which could cause incomplete transfers or swirling transfer patterns.
10. Place the gel holder in the buffer tank so that the black panel of the holder is facing the black cathode electrode. Repeat for 2nd blot if necessary.
11. Fill the tank with transfer buffer to just above the level of the top row of circles on the gel holder cassette.
12. Place stir bar and heat sink in the tank.
13. Set the buffer tank on top of a magnetic stirrer at 4°C.
14. Turn on the stirrer and close the lid gently but firmly. Be sure that the electrode wires are connected to the correct pins. Plug the unit into a power pack
15. Turn on power to initiate transfer: For SDS gels and nitrocellulose membranes use 100V, 250mA for 2hours (alternatively run @30V, 40mA overnight)
16. Pour off the transfer buffer into hazard waste container for methanol.
17. Carefully but quickly, remove blot from the transfer apparatus and cut on clean surface into strips, using the marker as guides, so you have one strip of your protein and one for your loading control (e.g., actin, ~45 kDa). Place each strip in separate small container.
18. Block non-specific sites by adding Blocking Buffer (5%Milk 0.05% Tween in PBS) to containers, hold for **1 hour** at room temperature with gentle shaking.
19. Dilute primary antibody to correct concentration in sufficient blocking buffer (usually 1:1000)
20. Remove the blocking reagent and add the appropriate primary antibody dilution. Incubate **overnight at 4°C** on rocker.

Next day:

1. Prepare 250mL Washing Buffer: 10× TBS(25mL) with 0.3% Tween-20 (0.75mL)
2. Wash membrane by suspending it in Wash Buffer and shaking for ~1 hour. Replace buffer 4-6 times and repeat wash. Increasing the Wash Buffer volume and/or the number of washes may help reduce background.
3. Incubate blot with the appropriate HRP-conjugate dilution for 1 hour at RT with shaking in 10mL blocking buffer
4. Wash membrane as in step 2
5. Prepare Developing solution by mixing equal parts 125uL of A into 5mL of B (kept in 4C)

6. Incubate blot with Developing Solution on way to Phosphoimager
7. Remove blot from working solution and place it on Fluorescent stage
8. Image Blot