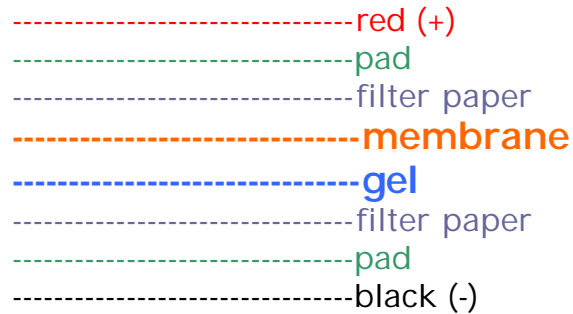


Western Blot Protocol

- Need to start with 20ug of protein, adjust the volume of each sample to 10ul with water or PBS
- Loading buffer = Laemmli sample buffer (BioRad #161-0737) +5% beta-mercaptoethanol (BME) (BioRad #161-0710)
For example: 95ul Laemmli +5ul BME
- Add 10ul sample loading buffer to each sample for a total volume of 20ul, mix well
- Place samples in boiling water for 5 minutes
**Both BME and heat act in denaturing the proteins, this is a completely denaturing electrophoresis (separate the proteins by their size)
- Flash centrifuge the samples after boiling, they are now ready to load
- Polyacrylamide gel – BioRad Ready Gel – gradient of gel depends on the size of the protein being isolated. Gel is Tris/HCl, SDS-PAGE. Open package with a razor blade. 29kD protein→12% gel
- Electrophoresis cell – put gel into the tray – make sure the rubber and the edge of the gel line up. Use a buffer dam if only running one gel. Close up the doors on the tray and place into cell. Fill the tray up with running buffer* (just on the inside) – watch for leaks. When you're sure there aren't any leaks, remove comb, fill the inside of the tray completely with buffer (cover wells) and cover the electrodes with buffer.
*Running buffer – 10x Tris/Glycine/SDS (BioRad #161-0732)
- Load molecular weight standard – Kaleidoscope Prestained Standards, Broad Range (BioRad #161-0324) – load 10ul on the negative side of the tray
- Load samples – use gel loading tips, load all 20ul
- Run gel for one hour at 120V (until the dye is all the way to the bottom)
- Remove gel from cell; use a razor blade to open the gel (cut along the white line on one side). Use finger nails to open the side up like a door (side that the comb was on)
- Need to have 2 pieces of paper cut the same size as your membrane (Trans-Blot Transfer Medium, BioRad #162-0146)
- Open gel; want gel to stick to the side with the comb!
- Attach one piece of filter paper – soaked in transfer buffer* - flip it over, attach membrane, add other piece of wet filter paper, smooth this out – no bubbles!!!!

- Place this between 2 pads (soaked in transfer buffer), close it up in the transfer cell (red/black)



- Cut the upper left corner of the membrane, this is where you should check to see if the marker has transferred.
- Transfer overnight at 26V or for one hour at 100V in cold room!
- Check for complete transfer (look at marker – make sure the largest has transferred)
- Wash membrane with PBS-T (0.05% Tween)
 - 0.05mL Tween + 99.95mL PBS
- Block membrane with 10% non-fat dry milk/PBS-T
 - 5g milk + 50mL PBS-T
- Make sure membrane is face up and add enough blocking solution to cover membrane – shake for 2 hours
- Pour off blocking solution, rinse with PBS-T
- Incubate membrane with primary antibody for 1 hour - shaking
 - 1:1000 dilution antibody in 5% milk/PBS-T
10ul Ab + 10mL 5% milk/PBS-T
- Wash with PBS-T, 3 washes with 15 minutes of shaking in between
- Incubate with second antibody (conjugated with horseradish peroxidase) for one hour (shaking)
 - 1:5000 dilution in 5% milk/PBS-T
5ul Ab + 25mL 5% milk/PBS-T
- Wash with PBS-T, 3 washes with 15 minutes of shaking in between
- Incubate the membrane with chemiluminescent substrate for 5 minutes in the dark.
 - SuperSignal (Pierce Chemical) – equal parts of each, make about 10mL
- Remove excess moisture – blot, do not rinse.
- Wrap in plastic wrap
- Apply the membrane for different amounts of time on a Kodak film in the dark chamber (30sec., 1 min., 5 min., 15 min., 30 min.)
 - Turn up a corner of the film so you know the orientation
- Develop film