# Western Blot – Complete Protocol

## Gel Casting

- Make 10ml gel mix for one small gel. Percent gel depends on size protein you are looking for. See Maniatis table for this and exact recipe for the percent gel needed.
- Use 1.5mm thick (thickest we have) spacers, along with thickest comb. Clean plates with EtOH, put in casting apparatus with spacers. Insert comb and mark with Sharpie ~1cm below comb bottom; remove comb.
- Prepare gel in 15 ml conical in following order – water, Tris pH 8.8, 30% acrylamide mix, 10% SDS, TEMED, 10% APS. Invert to mix, avoiding air bubbles.
- Load between plates to just above the Sharpie mark using a transfer pipette.
-Layer water-saturated butanol above gel to cause horizontal polymerization, using the top layer (butanol) in the saturated butanol solution. Gel is polymerized when a second interface forms between the butanol and gel.
- Pour off butanol, rinse with water. Use small piece Whatman paper to dry out between the plates.
- Prepare 5ml stacking gel in the same order as the resolving gel. Recipe in Maniatis. Be sure to use pH 6.8 Tris!
- Pour stacking gel over polymerized resolving gel. Insert comb, centering and making sure the top of the wells lines up with the top of the gel plate. Allow to polymerize.

## Prepare Samples for Gel

-Use Jennifer's Western Template to calculate amount of protein extract and 4X Sample Loading Buffer to mix.
-Mix extract with STB, boil for 5 minutes.
-Put on ice to cool, but not so long the SDS precipitates.

## Running the Gel

- Remove gel comb, remove gel from casting tray, mark the bottom of wells with Sharpie, and put into gel rig.
- Add ~1L 1X Running Buffer with SDS, enough to cover gel. Use 100ml 10X running buffer (15g Tris Base, 72g glycine per 500ml at pH 8.4), 10ml 10% SDS (10g SDS in H2O to 100ml), and 890ml distilled water.
- Briefly spin samples to get all of the volume to the bottom.
- Load 5ul protein ladder in first lane you will use, trying to avoid the two lanes on the end of the gel.
- Load full sample volume in appropriate wells
- Run at 80V until samples are into the resolving gel. At this point, voltage may be turned up.
-Watch protein marker migration to determine how long to run the gel.

## Transferring

-Pour buffer out of gel rig, remove gel
-Remove one spacer; use it to pry the plates apart
-Use removed plate to cut stacking gel off top and loading dye off bottom, also notch lower right corner

-Cut 2 pieces Whatman paper and 1 piece of membrane (kept in refrigerator). Notch bottom right corner of membrane
-Prewet membrane in small dish with 1X transfer buffer; pour enough 1X transfer buffer into dish with transfer sandwich apparatus to cover when all layers of sandwich are assembled. Also pour some 1X transfer buffer into transfer rig with small stir bar in it.
-Prepare sandwich – black side on bottom of dish, layer of sponge, whatman paper, gel (must flip backwards so will transfer in proper orientation, i.e. notch ends up in lower left), membrane (same orientation as gel), whatman paper, sponge, close. Be sure there are no air bubbles as you add each layer, and hold layers together as working until it is closed.
-Place sandwich in transfer rig with the black side of sandwich facing black side of rig. Fill rig with the transfer buffer from the two dishes, so that paper is wet but enough room at top that it won't spill walking down the hall.
-Bring to cold room, get stir bar stirring. Run at 30 mA overnight. If not transferring in cold room, place ice block in rig to keep buffer cool.

10X transfer buffer – 0.2M Tris base, 1.5M glycine – 24.44g Tris, 112.60g glycine in 1L H2O. Store in cold room.

1X Transfer buffer – 100ml 10X buffer, 200ml MeOH, 700ml H2O – Can be reused a few times, until turns yellow or lose too much volume. Store in cold room.

Blocking and Antibody Incubation
-Pour out transfer buffer (can be reused several times), remove membrane from sandwich.
-Put membrane in small dish, add some Ponceau stain, pour off after brief (less than minute) incubation on bench. Ponceau can be reused indefinitely.
-Rinse Ponceau from membrane with dH2O. Will be able to see if proteins loaded approximately equally and if there were any air bubbles in the transferring sandwich (spots where protein didn't transfer). Xerox the membrane for notebook.
-Incubate on rocker in ~25ml Blocking Buffer for one hour. Blocking buffer is TBST 5% milk (2.5g milk/ 50ml TBST). TBST is 100ml 10X TBS, 1ml Tween 20, and water to 1L.
-Make primary antibody to proper dilution in 15ml TBST 5% milk. For active caspase 3, need 1:1000 dilution, so 15ul in 15 ml. Pour off blocking buffer, and then add the diluted primary antibody. Rock for proper amount of time. For this antibody, it is 2 hours.
-Wash membrane 3 times for 5 minutes each with TBST.
-Incubate membrane with HRP-conjugated secondary antibody – 1:5000 dilution in TBST 5%milk (5ul Ab in TBST milk). In this case, it is goat anti-rabbit.
-Wash 3 times for 5 minutes each with 15ml TBST.

Detection
-During final washes, put an aliquot of ECL reagent (in large FACS tube, Daisy freezer) at 37C to thaw.
-Remove an aliquot of second ECL reagent (small eppie) from freezer. Gather everything you will need for rest of process - hydrogen peroxide, pipetman 10 and 200, tweezers, film, film cassette, saran wrap, timer.

-Place saran wrap on closure side of cassette.  Pour wash off of membrane.
-Add 30ul hydrogen peroxide and 10ul second ECL reagent to the thawed FACS tube of reagent, mix by inversion, and pour over membrane.
-Agitate membrane for ~ 1 minute, but not much longer.
-Using tweezers, remove membrane and dry somewhat on paper towel.  Move membrane to film cassette.
-Close and open cassette, dry any fluid that has seeped out around saran wrap, bring cassette, timer and film to dark room.
-Turn on developer to warm up.
-In dark, remove a piece of film and crease in bottom right corner, then place on top of saran wrap over film and close.  Start timer.  Try 2 minutes if don't know how signal will be.  Best time for this particular blot was 5-10 seconds.  Be sure to put film back in box, open side down, before turning on light.
-After correct time on membrane, in the dark, remove film and insert in developer.  Film must be flat against side of tray, short side in first.  It is safe to turn on the light when green light on the developer goes off.
-Reexpose film for different length of time if necessary.  Do reexposures as soon as possible because the chemiluminescence decreases rapidly.
-After films are developed, be sure to mark molecular weight standards on film.