Protocol: Western Blots with Tris-tricine Gels

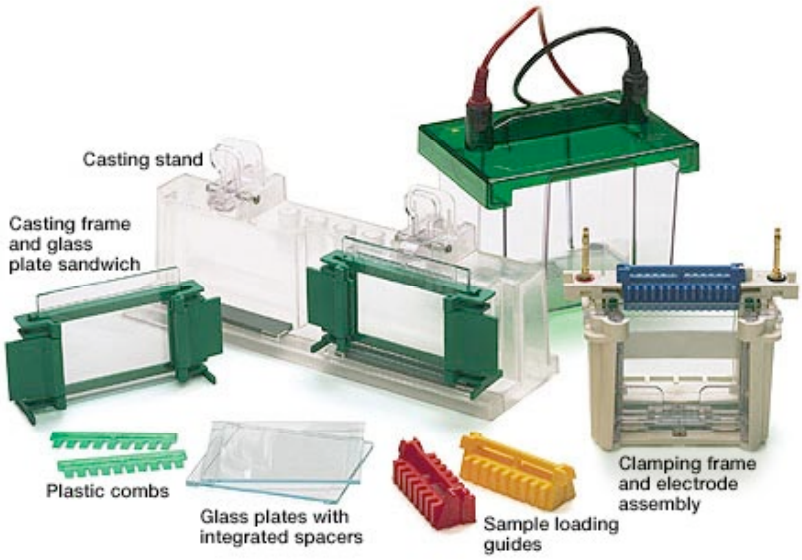
Isolate protein:

For adherent cells in a 6-well plate:

1. Remove media with vacuum.
2. Wash cells 1x with TBST.
3. Lyse with 350 µL IP lysis buffer (with 1% NP-40 and Sigma Protease Inhibitor Cocktail 1:100) or 350 µL TNE lysis buffer (with Sigma PIC 1:100) using rubber policeman.
4. Transfer lysate to eppendorf tube on ice.
5. Incubate on ice 30 min, vortexing thoroughly every 10 min.
6. Centrifuge at 14,000 rpm for 15 min.
7. Transfer supernatant to fresh eppendorf tube on ice.
8. Quantitate amount of protein using Bio-rad D_c Protein Asssay (similar to Bradford).
9. Add 2x tris-tricine sample buffer.
10. Boil 5 min.
11. Store at -20° C or proceed to Western blotting.

Casting the gel: (SEE Tris-Tricine Gel and Buffer Recipes)

1. Thoroughly clean glass plates prior to casting the gel. (I wipe them down with 70% EtOH.) Set up glass plate sandwich in gel casting stand. (We use Bio-rad Mini PROTEAN 3 Electrophoresis System).



2. In a conical vial, mix all components for separating and stacking gels except for 30% APS and TEMED.
3. Add 30% APS to separating gel. Mix by inverting a few times. Add TEMED to separating gel. Mix by inverting a few times. Gel will begin to polymerize now! Work quickly!
4. Pipette gel into glass sandwich until solution is about ¾ of the way to the top. Overlay with about 1 mL dH_20. Allow to polymerize. (You can use the remaining gel solution in the conical vial as a guide to determine when it's polymerized. It usually takes about 30-45 min.)

5. After gel has polymerized, dump off water. Remove all water by placing a Kimwipe in the corner of the sandwich.
6. Add 30% APS to stacking gel. Mix by inverting. Add TEMED to stacking gel. Mix by inverting.
7. Pour until gel reaches the very top of the glass plate. Insert comb.
8. Allow stacking gel to polymerize. (This takes 20-30 min.)
9. Proceed to running the gel or wrap the gel in damp paper towels and saran wrap and store in the 4° C fridge for up to 1 week.

Or you could skip all of this and buy pre-cast gels, but they really are very easy to make! (Bio-rad is a good resource for pre-cast gels.)

Run the gel:

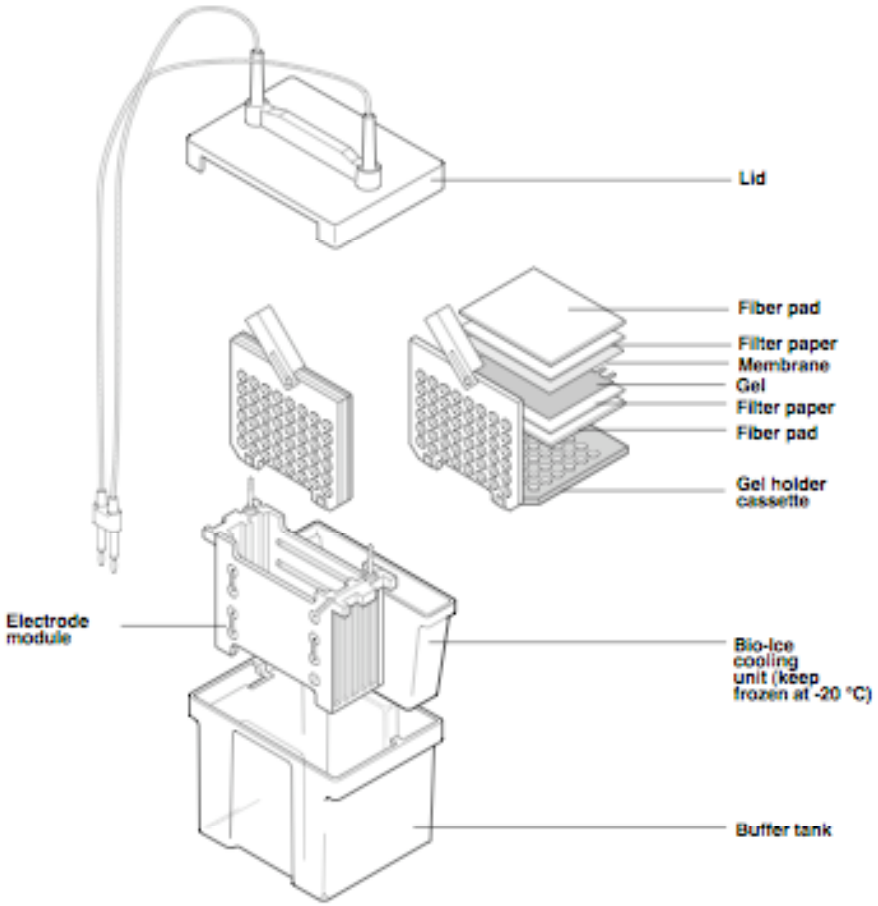
1. Place 2 gels (or 1 gel and 1 buffer dam) into clamping frame and electrode assembly.
2. Place in tank.
3. Fill inner chamber with 1X Tris/tricine/SDS running buffer. Make sure that this buffer is filled up above the top of the lower glass plate.
4. Fill outer chamber with 1X Anode buffer.
5. After boiling samples, load into wells. Use 5 µL of Bio-rad Precision Plus All Blue Standard. Load equivalent amounts of protein/well for samples. (20 µL will fit into each well of a 15-well comb. 30-35 µL will fit into each well of a 10-well comb.)
6. Run at 30V constant until samples have completely run through the stacking gel and are in the separating gel (about 1 hour). Run at 100V constant until ion front reaches the bottom of the gel (about 2 hours). (If you like, you can crank up the voltage to 175V constant and the gel will run faster.)

Transfer the gel:

1. For our transfers, we use the Mini Trans Blot Cell from Bio-rad.

2. Set up the transfer sandwich:
    a. Lay an open gel holder cassette on a few paper towels.

b. Thoroughly wash fiber pads with dH_20. Using forceps, dip into 1x transfer buffer and place 1 fiber pad onto each side of the holder cassette.
c. Dip 2 pieces of Whattman filter paper into 1x transfer buffer and place each piece on top of fiber pad.
d. Dip 1 piece of nitrocellulose membrane (cut to the size of your gel) into 1x transfer buffer and place on top of the Whattman paper on the white side of the cassette.



e. Dump running buffer from gel tank and remove one of the glass plates from your gel with a gel wedge.
f. Orient your gel over the membrane so that the marker is to the far left of the membrane and your samples are to the right of the marker. Gently use the gel wedge to pry the gel from the glass plate onto the nitrocellulose membrane. Use a glass pipette dipped in transfer buffer to smooth out the gel.

3. Place the sandwich into the electrode module with the white side of the gel cassette facing the red side of the electrode module (the black side of the cassette should be

facing the black side of the electrode module). Place the electrode module into the buffer tank.

4. Fill the tank with 1x transfer buffer.
5. Transfer at 4° C for 2 hours at 500 mA constant for 2 gels in 1 tank (or 350 mA constant for 1 gel in a tank).
6. After transfer is complete, remove cassette from tank. Throw away filter paper and the gel. Cut off any excess nitrocellulose membrane (the outer edges that the gel did not touch). Mark the ladder with a pencil. Place into a small box with a lid.

Western blot:

1. Block membrane for 1 hour at RT with 10 mL of 5% milk/TBST with gentle shaking.
2. Add primary antibody to blocking buffer (typically at a 1:1000 dilution, but check each antibody for optimal dilution). Incubate at RT for 1 hour or at 4°C O/N.
3. If you incubated for 1 hour at RT, wash 3X with TBST (5 min each) at RT with shaking. (If you incubated O/N, wash 1X for 15 min and 2 more times for 5 min each).
4. Incubate in secondary antibody (1:5000 in 5% milk/TBST) for 30 min at RT with shaking.
5. Wash 3-4 times with TBST, 5 min each, at RT with shaking.
6. In a fresh box, prepare detection reagents (Amersham ECL Western Blotting Detection Reagents). This will vary for each company's detection reagent, but for the Amersham ECL reagent, pipette 5 mL reagent 1 to the far left side of a box and 5 mL of reagent 2 to the far right side of the box. Do not allow the reagents to touch until you're ready to add your blot.
7. Using forceps, place blot in box containing detection reagents. Mix so that entire surface of blot is covered. Incubate 1 min with shaking.
8. Remove blot from detection reagents with forceps.
9. Use a piece of Whattman filter paper to remove some of the excess moisture from the membrane.
10. Place blot onto a sheet of saran wrap.
11. Fold the saran wrap over the blot so that no bubbles are visible between the blot and the saran wrap.
12. Add a glow-in-the-dark sticker to the top right corner of the saran wrap-blot. (Keep your stickers in the dark.)
13. Immediately develop your film:
    a. In the dark room with the lights off, expose the blot to film for various lengths of time (try 30s, 1 min, 2 min, 5 min)
    b. Run film through the processor.
14. After your film has run through the processor, place your blot behind the film and line up the stickers on the film and the blot. Use a sharpie to mark the ladder on the film. Also, write what lysates are in each lane and exposure times.