Western Blot Protocol

1) Turn on dry heating block to 100 °C (make sure it is heating).
2) Prepare 1x NuPAGE running buffer.
   a. 50 ml NuPAGE MES SDS Running buffer 20x + 950 ml milliQ water
   b. Set aside 200 ml for inner chamber – add 500 ul NuPAGE antioxidant no more than 30 minutes before electrophoresis
3) Set up one eppendorf per sample, label or arrange them in order to prevent confusion.
4) Prepare samples.
   a. Sample preparation depends on the gel size.
      
      | Gel Size   | Well Size | Loading Maximum | Prepare |
      |------------|-----------|-----------------|---------|
      | 1.0mm (10 wells) | 25λ.      | 23λ.            | ~30λ.   |
      | 1.5mm (15 wells) | 25λ.      | 23λ.            | ~30λ.   |

   b. 10x NuPAGE reducing agent (total/10 λ) + 4x LDS sample buffer (total/4 λ) + equal weight of protein per lane (from BCA analysis) + water (to achieve the proper total volume)
   c. Make up master mix of sample buffer, reducing agent, and H2O. Distribute into tubes and then ass samples.
5) Heat samples at 100 °C for 10 min.
6) Prepare electrophoresis equipment.
   a. Remove the NuPAGE gel from the pouch.
   b. Rinse the gel cassette with di water and peel off the tape from the bottom.
   c. Gently pull out the comb
   d. Orient the two gels in the mini-cell such that the exposed “well” side of the cassette faces inward. Use the plastic blank if you’re only running one gel.
   e. Lock gel assembly.
   f. Fill the inner buffer chamber with a small amount of the running buffer to check for tightness. If it leaks, discard the buffer, re-seal the chamber and repeat.
   g. Once the seal is made properly, fill the inner chamber with inner running buffer until the level is over the wells (~200 mL).
   h. Fill the outer chamber with 600 mL of x1 running buffer.
7) Prepare the ladders.
   a. 1/4 LDS sample buffer + 1/4 ladder + 1/2 x1 PBS
   b. Make/load the same amount as samples (above table).
8) Prepare blanks for empty wells.
   a. 1/4 LDS sample buffer + 3/4 x1 PBS
   b. Make/load the same amount as samples (above table).
9) After the samples have heated for 10 minutes, vortex and pulse spin in the centrifuge (~5 sec) to pull down the liquid.
10) Load the appropriate amount of each sample, blank and ladder into the gel using a p20.
11) Diagram gel layout.
12) Run electrophoresis.
a. For medium separation run at 100 V for 90 minutes or 200 V for 45 minutes. For high separation (running the blue dye off the gel completely) run at 100 V for 140 minutes or 200 V for 70 minutes.

b. Lower voltages give better results but take longer.

13) Make the Biorad transfer buffer.

   100 ml 10x Biorad transfer buffer (tris/glycine buffer)
   200 ml methanol
   700 ml water

14) Take the gels out of the cassette, cut off wells evenly and cut bottom of gel so no dye is left on the gel.

15) Soak the gel, membrane and extra thick filter paper (2 per blot) for ~5 min. Cut to fit the gel.

16) Assemble the components on the Biorad semi-dry transfer machine.
   a. Place filter paper down first, then the membrane, the gel, and finally the last filter paper. Roll out bubbles at every layer with a half pipette.

17) Transfer the gel:
   a. check to see what conditions are best
   b. for 1.0mm gel, run at 10V for 40-45 minutes.

18) Cut membrane appropriately.

19) Make 1:2 Odyssey blocking buffer (likely already diluted and stored at 4 °C).

20) Block membrane with 1:2 Odyssey blocking buffer for one hour at room temperature on shaker.

21) Prepare antibodies.
   a. Dilute in 1:2 Odyssey blocking buffer as per manufacturers suggestion (1:1000)
   b. These solutions can be used three total times before discarding.

22) Discard Odyssey blocking buffer and add primary antibody solution. Incubate overnight at 4 °C (on the shaker in the cold room).

23) The next day, remove primary antibody solution, and do 3 five-minute washes with PBS-Tween.
   a. 0.1% tween in PBS: 100 mL 10x PBS + 900 mL H2O + 1 mL Tween-20

24) Prepare secondary antibody.
   a. Dilute in 1:2 Odyssey blocking buffer as per manufacturers suggestion. (1:5000 for anti-rabbit)

25) Add secondary and shake at room temperature for two hours.

26) Discard secondary antibody solution. Repeat 3 five-minute washes with PBS-Tween.

27) Image on the Licor.

28) To re-probe:
   a. Shake at room temperature for 5 minutes in 0.2 M NaOH.
   b. wash well with milliQ water and then a PBST rinse
   c. Re-image on the Licor to see if any primary/secondary remains bound to the membrane.
   d. Repeat steps a-c until no signal is detected.
   e. Add primary antibody solution and incubate overnight at 4 °C (on the shaker in the cold room).

29) Repeat steps 23-28!

30) Once all probing is complete store the membrane in laboratory wrap at 4°C.