Lazzara Lab
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NuPage Western Blotting Protocol

1. Turn on dry heating block to 100ºC (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 µL** NuPAGE antioxidant no more than 30 min before electrophoresis.
3. Set up one eppendorf tube per sample, and label or arrange them in a rack in order to prevent confusion.
4. Prepare samples:
    a. Sample preparation depends on the gel size.

Gel Size	Well Size	Loading Maximum	Prepare
1.0 mm (10 wells)	25λ	23λ	~30λ
1.5 mm (15 wells)	25λ	23λ	~30λ

    b. 10x NuPAGE reducing agent (total/10 λ) + 4x LDS sample buffer (total/4 λ) + equal mass of total protein per lane (from BCA analysis) + water (to achieve the proper total volume)
    c. Make up master mix of sample buffer, reducing agent, and H_20. Distribute into tubes and then add to samples.
5. Heat samples in heat blocks 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 1x 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 1x PBS
    b. Make/load the same amount as samples (above table).
9. After the samples have heated for 10 min, 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 pipet.
11. Diagram gel layout in your lab notebook.
12. Run electrophoresis.
    a. For medium separation run at 100 V for 90 min or 200 V for 45 min. For high separation (running the blue dye off the gel completely) run at 100 V for 140 min or 200 V for 70 min.
    b. Lower voltages give better results but take longer.

13. Transfer the gel to nitrocellulose using either a wet tank transfer or semi-dry transfer:
    a. Wet tank transfer – see *Wet tank transfer protocol*
    b. Semi-dry transfer – see *Trans-Blot Turbo Semidry Blotting* protocol
14. Make 1:2 Odyssey blocking buffer (likely already diluted and stored at 4°C).
15. Block membrane with 1:2 Odyssey blocking buffer for 1 hr at room temperature on shaker.
16. Prepare antibodies.
    a. Dilute in 1:2 Odyssey blocking buffer per manufacturer's suggestion (e.g., 1:1000).
    b. These solutions can be used three total times before discarding. However, for publication data fresh solutions are recommended.
17. Discard Odyssey blocking buffer and add primary antibody solution. Incubate overnight at 4°C (on the shaker in the cold room).
18. The next day, remove primary antibody solution (retain in a conical in the fridge if this is second or first use), and do **3 5-min washes** with PBS-Tween.
    a. 0.1% Tween in PBS: 100 mL 10x PBS + 900 mL H_2O + 1 mL Tween-20
19. Prepare secondary antibody.
    a. Dilute in 1:2 Odyssey blocking buffer per manufacturer's suggestion (e.g., 1:5000 for anti-rabbit).
20. Add secondary and incubate at room temperature for 2 hr on shaker.
21. Discard secondary antibody solution. Repeat **3 5-min washes** with PBS-Tween.
22. Image on the Licor Odyssey scanner.
23. To strip antibodes and reprobe a blot:
    a. Shake at room temperature for 5 min in 0.2 M NaOH.
    b. Rinse well with MilliQ water and then do one PBS-Tween 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. Repeat steps 21-27!
24. Once all probing is complete, store the membrane in laboratory wrap at 4°C.