WESTERN BLOTTING (Proteins) **7/2005 Joanne Odden**

Stock solutions

1. **5X TBS – Frig.**
   100 mM Tris Base (12.11 g)
   2.5 M NaCl (146.0 g)
   Bring to 1.0 L with dH_2O, pH 7.5

2. **1X TBS – Frig.**
   5X TBS (200.0 ml)
   Bring to 1.0 L with dH_2O

3. **TBS/Tween – Frig.**
   5X TBS (60.0. ml) [20.0ml]
   Tween 20 (1.5 ml) [0.5 ml]
   Bring to 300.0 ml with dH_2O [for 100 ml]

4. **TBS/Milk/Tween – Frig.**
   5X TBS (60.0. ml) [20.0ml]
   Tween 20 (0.3 ml) [100 ul]
   Nonfat Dry Milk (15.0 g) [5.0 g]
   Bring to 300.0 ml with dH_2O [for 100 ml]

5. **Continuous Nitrocellulose Transfer Buffer** (see 2D Multiphor II manual) **– by Multiphor II**
   40 mM Glycine (2.93 g)
   48 mM Tris (5.81 g)
   0.0375% SDS (375.0 mg)
   20% Methanol (200 mL)
   Bring to 1.0 L with dH_2O

Protocols for Western Blotting

1. Run 1D PAGE gel
2. Transfer gel to Nitrocellulose Transfer Buffer and agitate for > 15 min in plastic dish.
   Note: can store gel in Transfer buffer overnight at room temp. (22-24 C).
3. After 15-20 min of soaking in Transfer Buffer, measure dimensions of gel in cm.
   Note: if < 15 min, gel is still shrinking and will give erroneous measurements.
4. Using gloves, cut 18 sheets of electrode filter paper and 1 sheet of nitrocellulose membrane (N-membrane) to fit gel exactly with paper cutter. **Note: If filter papers are larger than gel, a shorting bridge will occur and the proteins will not transfer correctly!**
5. Separate the 18 sheets of filter paper into two 9 sheet piles and place in Petri dishes.
6. If applicable, remove the 2D gel surface. Soak graphite electrodes with dH_2O soaked paper towels.

7. Individually soak 9 filter papers in Transfer Buffer and stack vertically on graphite anode. **Note: make sure there are no air bubbles trapped between layers of filter paper, N-membrane, or gel; otherwise, proteins will not transfer well!**
8. Soak N-membrane in Transfer Buffer and stack on filter paper.
9. Place PAGE gel on top of N-membrane.
10. Individually soak remaining 9 filter papers in Transfer Buffer and stack vertically on top of gel.
11. Insert graphite cathode and place glass plate over cathode to add more weight.
12. Replace Multiphor II cover and turn on power supply.
13. Multiply surface area (cm) of gel by 0.8 mA/cm^2 to get current setting.
14. Power supply settings:
    a. Program #1
    b. Use default settings on all other options but current (mA). Run for 1.0 hr.
    c. Change mA settings from Step 13 above.
    d. Once finished making settings, push "Run" to start system.
15. Once system is off, turn power supply off and remove lid.
16. Carefully remove graphite cathode and remove upper stack of filter paper.
17. Remove PAGE gel, notch one corner, and place in Coomassie stain overnight. Then destain to check for protein transfer.
18. Remove N-membrane and notch same corner as #17 for orientation.

Note: can be stored in dry ziplock bag overnight in frig.

Also note: All subsequent steps require room temperature and elevated agitation of the solutions around membrane.

19. Put membrane into some TBS/Milk/Tween for 1 hr in weight boat at 37◦C. **Note: can be stored overnight in frig in Petri dish.**
20. 15 min – TBS/Tween
21. 1-2 hr – **TBS/Milk/Tween** with primary antibody of a specific dilution
    -for sym32 and half of small protein gel: 2.5 ul anti-sym32 in 5 ml
    *This should be a more vigorous step (~5 setting for shaker)
22. 8-10 min 1X TBS
23. 8-10 min TBS/Tween
24. 8-10 min 1X TBS
25. 1 hr – Secondary antibody (ECL kit) in **1X TBS** (1:5000) **in a <u>new container</u>**
    -for sym32 and half of small protein gel: 1 ul anti-rabbit IgG from donkey (top shelf of frig side door in the middle)
26. Rinse as in steps 22, 23, and 24 above
27. Mix equal volumes of ECL Chemicals A and B into a clean container just enough to cover N-membrane. **Note: Do not cross contaminate reagent chemicals or they will not chemilluminesce anymore!**

    I have mixed 0.5 ml of each into a clean tube, then pip'ed the 1 ml onto the N-membrane while it is on Saran Wrap. ~JO
28. 1 min—Chemilluminescence solution from Step 27 above.
29. Remove N-membrane and blot excess chemicals from corner using KimWipes.
30. Wrap in Saran Wrap and tape into photographic cassette and expose X-ray film to get visual record of target protein. Take care not to feed the blot through the developing machine.