SOP: SP011.3
Modified 3/6/18 by MCL

Western Blot Protocol

Materials and Reagents:

1. Blotting Buffer (SOP R010)
2. 10% BSA in TBST
3. TBST (1.21g Tris, 8.77g NaCl, 0.5ml 100% Tween 80, q.s. to 1L with MilliQ H_2O, pH 7.4).
4. TBS (1.21g Tris, 8.77g NaCl, q.s. to 1L with MilliQ H_2O, pH 7.4)
5. Appropriate Primary and Secondary Antibodies
6. SigmaFast BCIP/NBP Tablets (Sigma # B5655)
   or
7. 4-Chloro-1-Naphthol Tablets (Sigma # C6788)
8. Milli-Q H_2O
9. Chromatography paper 10cm x 100m (Whatman # 3030 672) also called "filter paper"
10. 0.2um Nitrocellulose Membrane (Biorad # 9004-70-0)
11. Shaker Table
12. Completed SDS-PAGE gel (SOP SP007)
13. Supplies listed in Table 1

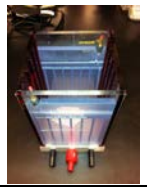
Power Supply with adapter	Blotting Cassette	Foam sponges	Transfer tank (Hoeffer TE22)	Transfer tank lid
				

Table 1 – Supplies needed for Western Blot Transfer

Protocol:

1. _____ Run an SDS-PAGE gel according to SOP SP007 with desired proteins or fractions.

2. _____ While gel is running, cut appropriately sized pieces (4 total) of Whatman chromatography filter paper.

3. _____ Cut nitrocellulose membrane to be slightly larger than the gel size (note 1).

4. _____ Open the blotting cassette and create a stack as follows:
   - 1-2 foam sponges
   - 2 sheets filter paper
   - Nitrocellulose (remove paper backing first)
   - 2 sheets filter paper
   - 1-2 foam sponges

5. _____ Close the cassette and place into the transfer tank.

6. _____ Fill the transfer tank with blotting buffer to the maximum fill line.

7. _____ Let the cassette soak in the blotting buffer until the membrane is thoroughly wet (at least 5-15 minutes).

8. _____ Once the SDS-PAGE gel is completed, remove the blotting cassette from the transfer tank and open it to expose the nitrocellulose (note 2).

9. _____ Apply a small amount of blotting buffer to the nitrocellulose to keep it wet while preparing your gel.

10. _____Use a gel knife to gently crack open the gel cassette, cut the wells and foot off the gel.

11. _____Carefully remove the gel from the plastic plate and wet it with blotting buffer

12. _____Place the SDS-PAGE gel face-up (with the ladder on the left) on top of the nitrocellulose.

13. _____Wet the gel with a small amount of blotting buffer from the tank.

14. _____Use a roller or serological pipette to gently smooth out the gel and remove any air bubbles between the gel and the membrane.

15. _____Place the remaining pieces of filter paper on top of the gel, add more blotting buffer, and roll out again to remove all air bubbles.

16. _____Close the cassette and place into the transfer tank (note 3).

17. _____Place the lid on the tank, with the red lead on the side with the nitrocellulose (usually the white side of the cassette), and the black lead on the side with the gel (the black side of the cassette) (note 4).

18. _____Plug the leads into a power supply, making sure that red is plugged into red and black is plugged into black.

19. _____Set the voltage to 50V for 1 hour, or 5-10V for 15 hours for an overnight transfer.

20. _____Once the transfer is complete, turn off the power supply and remove the cassette.

21. _____Carefully remove the top layers of filter paper.

22. _____With the gel still in place, cut off excess nitrocellulose with a sharp, clean pair of scissors.

23. _____Transfer the membrane to a small blotting container. Discard the gel and filter paper.

24. _____*Optional Ponceau S Staining* (note 5):
    - Cover the nitrocellulose with a small amount of Ponceau S stain
    - Place on a shaker table for 5-15 minutes
    - Decant the stain and rinse several times with TBST
    - Place on the shaker table until the background clears
    - If needed, scan or take a picture of the stained blot

25. _____Prepare blocking buffer by adding 1 ml 10% BSA to 9 ml TBST (note 6).

26. _____Add blocking buffer to the nitrocellulose.

27. _____Place blotting container on shaker table and let incubate for at least 1 hour at room temperature or overnight at 4°C (note 7).

28. _____Pour off the blocking buffer and rinse the membrane three times briefly with TBST, then for 5 minutes on the shaker.

29. _____While the membrane is rinsing, dilute the primary antibody to the proper titer in 10 ml TBST (note 8).

30. _____After rinsing the membrane, apply the primary antibody and incubate for at least 1 hour at room temperature or overnight at 4°C.

31. _____Pour off primary antibody and rinse the membrane three times briefly with TBST, then for 5 minutes on the shaker table.

32. _____Prepare secondary antibody at a 1:2500 titer in TBS (4 µl in 10 ml) (note 9).

33. _____After rinsing, apply the secondary antibody and incubate at room temperature for 30-45 minutes (note 10).

34. _____After incubation with the secondary antibody, rinse the membrane three times briefly with TBS (not TBST), then for 5 minutes on the shaker table.

35. _____Based on the secondary antibody used, prepare the appropriate substrate as follows:

- Alkaline phosphatase (AP) conjugated antibody → use SigmaFast Developer
  - Dissolve one SigmaFast developer tablet (stored at -20°C) in 10 ml of Milli-Q water. Bath sonicate and/or vortex until dissolved.
- Peroxidase (HRP) conjugated antibody → use 4-Chloro-1-Naphthol Developer
  - Dissolve one 4-Chloro-1-Naphthol tablet (stored at -20°C) in 10 ml methanol
  - Add 2 ml of methanol stock solution to 10 ml TBS
  - Add 5 µl 30% hydrogen peroxide immediately prior to use

36. _____Apply the appropriate developer solution to the membrane and watch for the blot to develop.

37. _____Once your band of interest appears, pour off the developer solution and rinse with Milli-Q water several times to stop the reaction.

38. _____Place the blot on a paper towel and fold the towel to cover the blot.

39. _____Once dry, the blot can be placed in a notebook, or scanned.

Notes:

1. Be sure to handle the nitrocellulose with gloves and tweezers. Touching any part of the nitrocellulose membrane even with gloves may result in some undesired background.
2. The blotting cassettes and transfer tank are fully reversible, so it is important to always be aware of which side of the cassette the nitrocellulose is on. It is helpful to assemble the cassette in the same way every time (for example, always with the nitrocellulose on the white side).
3. If you are transferring more than one western blot, be sure that all of the cassettes are facing the same direction in the tank.
4. Current will run from the negative lead (black) to the positive lead (red). This will drive the current towards the nitrocellulose and force the proteins from the gel onto the nitrocellulose (i.e. **"run to red"**).
5. Ponceau S will reversibly stain all proteins on the nitrocellulose to confirm the efficiency of transfer. The stain is made as follows:
   - 0.1% Ponceau S (Sigma # P-3504) – 0.1 g
   - 5% Acetic acid – 5 ml
   - MilliQ water – qs to 100 ml
6. Generally 1-3% BSA is used to block. Some antibodies may require a different blocking buffer, such as non-fat dry milk, for optimum results.
7. If Ponceau S was used, the blocking buffer may need be changed after 15 minutes to remove excess stain.
8. Each primary antibody has an optimized titer. Check product label/information for recommended western blot titer.
9. Ensure that you are using the appropriate secondary antibody for your primary (anti-mouse secondary for mouse monoclonal, anti-rabbit secondary for rabbit polyclonal, etc.)
10. Incubating in secondary antibody for too long may increase non-specific binding.

References:

http://www.hoeferinc.com/downloads/TE22-IME0.pdf

Antibodies: A Laboratory Manual. Ed Harlow and David Lane. Cold Spring Harbor Laboratory, New York. 1988. pp. 471-510.