SOP: SP011a
Modified 9-12-19 by MCL

Western Blot Protocol
Using Invitrogen Mini Blot Module

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

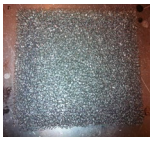
Power Supply with adapter	Blotting Cassette	Foam sponges	Transfer tank (Hoeffer TE22)	Transfer tank lid
				

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 (2 total) of Whatman chromatography filter paper and nitrocellulose membrane (note 1).

3. _____ In a glass tray, pour 50-100 ml blotting buffer and soak two sponge pads as well as the filter paper and nitrocellulose (with paper backing removed). Squeeze the sponges several times in the liquid to remove air bubbles.

4. _____ Place the Cathode Core on a flat surface and add ~10 ml botting buffer to the surface.

5. _____ When gel is ready, create a stack as follows, using a roller to remove bubbles between each layer:
   - Sponge pad
   - Filter paper
   - Gel – place face down (with ladder on the right) to maintain left-to-right orientation
   - Nitrocellulose
   - Filter paper
   - Sponge pad

6. _____ Place the Anode Core on top of the stack and press the module assembly closed.

7. _____ Place the assembly in the Mini Gel Tank (remove cassette clamps from tank first) so that the electrodes on the Cathode Core are in contact with the cathode bar.

8. _____ Pipet blotting buffer from the glass tray to the inside of the module core to completely submerge the sponge pads. Do not fill past the gasket (note 2).

9. _____ Add di-H2O to the tank outside the module core up to the level of the cathode bar (~225 ml)

10. _____Place the lid on the tank and plug the leads into a power supply, matching **red**-to-**red** and **black**-to-**black** (note 3).

11. _____Set the voltage to 10 V for 1 hour (note 4).

12. _____Once the transfer is complete, turn off the power supply and remove the lid.

13. _____Remove the module core and pour the blotting buffer into the Used Blotting Buffer container.

14. _____Open the module and carefully remove the top sponge pad and filter paper.

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

16. _____Transfer the membrane to a small blotting container.

17. _____Discard the gel into a hazardous waste container and discard the filter paper into the trash. Rinse Mini Gel Tank, Blot Module, and sponge pads thoroughly with di-H2O.

18. _____*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

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

20. _____Add blocking buffer to the nitrocellulose.

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

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

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

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

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

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

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

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

29. _____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

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

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

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

33. _____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. Any remaining blotting buffer should be poured into the Used Blotting Buffer container. Blotting buffer can be used up to six times, and should then be discarded as hazardous waste.
3. Ensure that the power supply port that you are using has adapters installed that fit the Mini Gel Tank electrodes.
4. For faster transfer, try 15 V for 30-45 min (there may be some loss of sensitivity). Do not exceed 30 V. If overnight transfer is needed, the Hoeffer TE22 transfer tank should be used.
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.