SOP: SP011.4
Modified 6.20.2023 by KCB

Western Blot Protocol

Materials and Reagents:
1. Blotting Buffer (Western Transfer 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 Bottom (Negative) and Top (Positive)	Foam sponges	Transfer tank and Lid (Invitrogen)

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 chromatography filter paper.

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

4. _____ While the gel is still running, soak the foam sponges and filter paper blotting buffer using a glass Pyrex dish.

5. _____ Once the gel is finished running, rinse the tank and gel with DI water, and let the gel cool if it is warm to the touch. (Note 2)

6. _____ Using a gel knife, carefully crack and open the gel and trim the foot and wells of the gel.

7. _____ Open the blotting cassette and, ***starting with the cassette half labeled negative (-)***, create a stack as follows:
    - 1 foam sponge
    - 1 sheet filter paper
    - SDS-Page Gel
    - Nitrocellulose (remove paper backing first)
    - 1 sheet filter paper
    - 1 foam sponge

    If the stack is assembled on the wrong side of the cassette, the protein in the gel will be pulled into the blotting buffer and lost instead of onto the nitrocellulose. (Note 3)

8. _____ Close the cassette and place into the transfer tank. The tank can be run with either one or two cassettes.

9. _____ Fill the inside of the cassette with blotting buffer until full

10. _____Fill the outside tank with DI water to the fill line.

11. _____Place the lid on the tank with the wires facing to the back, making sure that each of the 4 metal pegs of the lid are fully inserted into the tank.

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

13. _____Set the voltage to 10V for 1 hour and press the run button.

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

15. _____Carefully remove the top layer of filter paper and sponge.

16. _____With the gel still in place, cut off excess nitrocellulose with a sharp, clean pair of scissors. (if the nitrocellulose wasn't already cut to size)

17. _____Transfer the nitrocellulose membrane to a small blotting container. Discard the gel in gel waste, and discard the paper.

18. _____*Optional Ponceau S Staining* (note 4):
    - 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 5).

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 6).

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 7).

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. (note 8)

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 (note 11):

- 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. Though not necessary, the gel is much less likely to break when cool. Attempting to cut the gel and place it in the cassette when still warm could result in a tear or break.
3. The blotting cassettes are not reversible. Make sure to always start assembling the 'sandwhich' with the negative side of the cassette as the base, or else the protein will run into the blotting buffer and be lost without transferring to the nitrocellulose.
4. 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
5. 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.
6. If Ponceau S was used, the blocking buffer may need be changed after 15 minutes to remove excess stain.
7. Each primary antibody has an optimized titer. Check product label/information for recommended western blot titer.
8. Most primary antibodies can be collected back into a 15 mL falcon tube to be reused up to 3-6 times depending on the clone, as to not waste primary antibody.
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.). The last letters on the end of an antibody lot will provide information on what secondary antibody to use. For example, ".mm" indicates that the antibody

is a “mouse monoclonal” antibody and thus the secondary antibody to use would be an anti-mouse secondary. “.rp” indicates a “rabbit polyclonal” antibody and would require an anti-rabbit secondary antibody.

10. Incubating in secondary antibody for too long may increase non-specific binding.
11. The developer to use is usually indicated on the vial of secondary antibody, such as “AP conjugated” (alkaline phosphatase conjugated) or “HRP conjugated” (horseradish peroxidase conjugated).

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.