SOP: SP070

Fluorescent Western Blot Protocol

Materials and Reagents:

1. Blotting Buffer (SOP R010.1)
2. ECL Advance Blocking Reagent (GE cat# RPN418V) prepared at 5% 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 Antibody
6. Appropriate Secondary Antibody – either ECL Plex Goat anti-rabbit IgG, Cy5 (GE cat #PA45011V) or ECL Plex Goat anti-mouse IgG, Cy2 (GE cat#28901108V)
7. Methanol
8. Milli-Q H_2O
9. Chromatography paper 10cm x 100m (Whatman # 3030 672) also called "filter paper"
10. 0.2 m Hybond-LFP PVDF transfer membrane (GE cat# RPN2020LFP)
11. Blotting Cassette, complete with foam pads
12. Transfer tank (Hoeffer TE 22)
13. Transfer tank cover
14. Foam sponges
15. Blotting cassette
16. Disposable pipet
17. Shaker Table
18. Power Supply with adapter
19. Completed SDS-PAGE gel (SOP SP007.1) --- (Note 1)
20. ECL Plex Fluorescent Rainbow Marker (GE cat# RPN850E)
21. Typhoon Imager (SOP SP071)

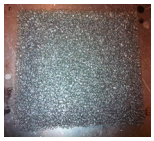
Power Supply with adapter	Blotting Cassette	Foam sponges	Transfer tank	Transfer tank cover
				

Table 1 – Supplies needed for Western Blot Transfer

Protocol:

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

2. _____ While gel is running, cut appropriately sized pieces of Whatman chromatography filter paper. Use a total of 4 sheets.

3. _____ Cut out an appropriate sized piece of PVDF membrane. Be sure to handle the PVDF with gloves and tweezers. Touching any part of the PVDF membrane even with gloves may result in some undesired background.

4. _____ Soak PVDF membrane in methanol for 20 sec, followed by Milli-Q water for 20 sec and transfer buffer for a minimum of 5 minutes.

5. _____ Place 1-2 sheets of the filter paper on top of one of the foam sponges on the white side of the cassette (Figure 1&2).

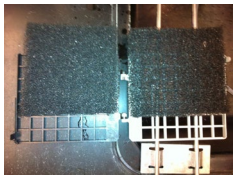


Figure 1 – Opened cassette with foam sponges.

6. _____ Place the other 1-2 sheets of filter paper on the foam insert on the black side of the cassette.

7. _____ Place the pre-soaked PVDF membrane on top of the filter paper on the white side of the cassette.

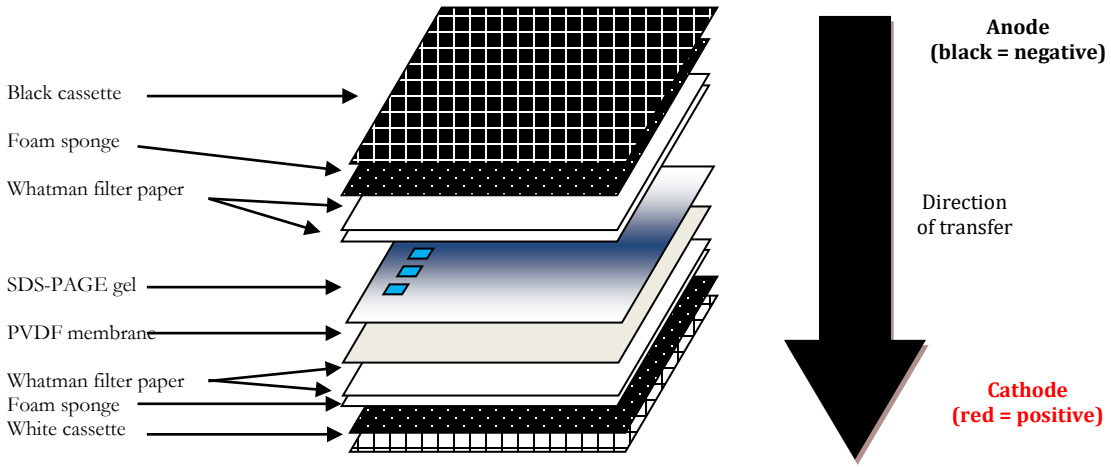


Figure 2 – Cassette order in Western Blot Transfer

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

9. _____ Fill the blotting chamber with blotting buffer to the maximum fill line on the side of the transfer tank to pre-wet the cassette.

10. _____ Let the cassette soak in the blotting buffer until the membrane is thoroughly wet.

11. _____ Once the SDS-PAGE gel is completed, remove the Western Blotting cassette from the transfer chamber and open it so that the white side of the cassette has the membrane.

12. _____ Carefully remove the gel from the plastic plate and dip it into the blotting buffer

13. _____ Place the SDS-PAGE gel on top of the membrane with the marker on the left side of the membrane.

14. _____ Wet the gel with a small amount of blotting buffer from the chamber.

15. _____ Carefully smooth the gel with a disposable pipette to remove any air bubbles between the gel and the membrane.

16. _____ Place the filter paper from the black side of the cassette over the gel and remove all air bubbles once more. Place one sponge on top of this and close the cassette.

17. _____ Place the cassette back into the blotting chamber.

18. _____ Place the lid onto the chamber, making sure that the positive lead (red = cathode) is on the side of the chamber that the white side of the cassette is facing. The negative lead (black = anode) must likewise be on the side that the black side of the cassette is facing. This will drive the current towards the white side of the cassette and force the proteins from the gel onto the membrane (i.e. "run to the red").

Figure 3 – Assembled Transfer tank

19. _____ Plug the leads into a power supply with adapter and set the voltage to 50V for 1-1.5 hours, or 5V for 15 hours for an overnight transfer.

20. _____ Once the transfer is complete, turn off the power supply and remove the cassette and open.

21. _____ Prepare blocking buffer by adding 0.2 g of the blocking buffer reagent to 10 mL of TBST.

22. _____ Transfer the membrane to the small blotting container with blocking buffer. Discard the gel and filter paper.

23. _____ Place blotting container on shaker table and let incubate for at least 1 hour at room temperature or overnight at 4 ˚C.

24. _____ Pour off the blocking buffer and rinse the membrane two times with TBST for 5 minutes on the shaker.

25. _____ While the membrane is rinsing, dilute the primary antibody to the proper titer in TBST (table 2).

26. _____ After rinsing the membrane, apply the primary antibody.

27. _____ Incubate with primary antibody for at least 1.5 hours at room temperature (Note 2).

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

29. _____ After rinsing the membrane, apply the secondary antibody diluted 1:2500 in TBS (Note 3) (table 2).

Primary Antibody	Secondary Antibody
Mouse monoclonal antibody	Goat anti-mouse IgG whole molecule, Cy2
Rabbit polyclonal antibody	Goat anti-rabbit IgG whole molecule, Cy5

Table 2 – Examples of Primary and Secondary Antibodies

30. _____ Incubate with secondary antibody for 1 hour, wrap blotting container in foil to shield the antibody from light. Too long of an incubation may increase the non-specific binding.

Table 3 – Detection of Fluorescence

31. _____ After incubation with the secondary antibody, rinse the membrane three times briefly with TBS (not TBST), then for 5 minutes four additional times while protected from light on the shaker table. (Note 4)

32.____ Detect the secondary antibody signal by scanning the membrane using a fluorescent laser scanner, such as the Typhoon Imager (in room B319).

Notes:

1. SDS-PAGE must be run with ECL ladder in order to detect with Typhoon imager
2. Two primary antibodies can be incubated simultaneously, however for multiplex detection to work, one primary must be mouse and the other must be a rabbit antibody.
3. Preparation of the fluorescent secondary antibodies must be performed in minimal light.
4. At this point a second secondary antibody can be added for multiplex detection.

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.