

Western Blot Protocol

1. Note gel gradient percentage (normally 8-16%).
2. Samples containing multiple proteins require 10-15 ug of protein per well. Purified samples containing single or very few proteins require less (2-5 ug). Less is required for the more sensitive process of silver stain (1 ug).
3. Dilute the samples at a 1:2 ratio with buffer and boil along with the appropriate marker for five minutes.
4. Load samples into the wells (~40 uL).
5. The module tank is filled with 1xTGS (inside at a higher level than the outside).
6. Run the gel (large criterion) at 100 V for 10 minutes then 160 V for 65 minutes (Program 8). For small gels run the gel at 40 V for 10 minutes then at 120 V for 1 hr.
7. To prepare for the trans-blot, soak the PVDF membrane in methanol for 1 minute, and then place it in the "sandwich" chamber with 2 fiber pads and 2 filter papers all soaking in **old** transfer buffer. The "sandwich" should be black side down.
8. After the gel has run, rinse it in **old** transfer buffer for 5-10 minutes on the shaker.
9. The sandwich is black side, fiber pad, filter paper, gel, membrane, filter paper, fiber pad, and red side. After each layer roll out any air bubbles which may have formed.
10. Run the transblot at 4° (in the cold room) using an ice pack with **new** transfer buffer. Running times can vary from 100 V for 1 hr, 90 V for 100 minutes or 40 V O/N. Depending on transfer buffer quality and/or experiment.
11. After the trans-blot block the membrane (facing up) with 5% nonfat dry milk in TBS-T (1g/20mL) for 1 hr., on the shaker, at room temperature. (can be O/N @ 4°)
12. Incubate with a primary antibody in blocking buffer O/N (can be 1 hr @ RT) @ 4° on a shaker.
13. Wash the membrane with TBS-T: 2x 5 minutes, 2x 10 minutes.
14. Incubate with a secondary antibody in 1-2% BSA prepared with TBS-T for ~1hr at RT.
15. Rinse with TBS-T: 2x 5 minutes, 2x 10 minutes, 4x 15 minutes.
16. Prepare 2 tubes: in each tube add 18 mL H₂O, 2 mL 1M Tris HCL (pH 8.3)
In tube 1/A: add 200 µL Luminol and 80 µL pCA (located in -80°)
In tube 2/B: add 120 µL 3% H₂O₂
17. Proceed to the Darkroom with the following items:
 1. The freshly prepared tubes 1/A and 2/B
 2. Timer
 3. Film Cartridge
 - 4.

5. 2 Filter papers
6. Forceps
7. Bag for membrane
18. Container to hold the membrane
19. Place it on top of the membrane
20. Close the cartridge for a development time of 1 minute.
21. Feed film into the machine. Wait for the film.
 8. Reduce development time in
22. Pour both tubes 1/A and 2/B into the container holding the membrane and then agitate for 1 minute.
17. With the forceps, remove the membrane and place it on the filter paper, then sandwich the membrane with the other filter paper in order to absorb any excess liquid.
23. Put the membrane, brighter band facing up, vertically inside the bag.
24. Place it inside the cartridge.
25. **Turn off the lights.**
26. Pull out film from the box. order to reduce the background.