How to immunoblot proteins
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1. Electrophorese protein samples on SDS-acrylamide gel.

2. Transfer the proteins to PVDF membrane (Immoblon-P, Millipore) at 800mA for 3 hours (2 hours is enough for most proteins) with Genie Electrophoretic Blotter (cat#4015, IDEA Scientific).

   Transfer buffer: 384 mM Glycine, 50mM Tris, 20% Methanol.
   (28.8 g Glycine, 6.05g Tris, 200 ml methanol, make up to 1L buffer)

   Wet the PVDF membrane in 100% methanol for a few seconds, then soak the membrane in transfer buffer.

   Order of transfer apparatus (bottom to top):
   Negative electrode > screen (rough side down) > sponge > filter paper > gel > membrane > filter paper > sponges >screen (rough side up) > positive electrode

3. Remove the blot after transfer and soak the membrane directly in 100% methanol for 1 min, remove the membrane and allow it dry at room temperature for 10 min.

4. Incubate the dry membrane directly into antibody solution containing PBS, 5% nonfat dry milk, 0.3%Tween 20 for one hour. The volume you need depends on the size of the membrane. You may also incubate the membrane in the solution without the antibody for 30 min before adding the antibody.
   The antibody dilution to use needs to be determined for each antibody and time of the incubation can be longer depending on the sensitivity of the antibody.

5. Place the membrane in a larger box, rinse with PBS-T (PBS with 0.3% Tween 20) first. Wash membrane in fresh PBS-T. 1 X 15min, 2 X 5min.

6. Incubate the membrane in secondary antibody (Horse radish peroxide conjugated) diluted in PBS, 0.3% Tween 20, 5% nonfat dry milk for 20 min (Goat-anti-Rabbit) or 1 hour (Goat-anti-Mouse) at room temperature.
   Secondary antibody dilution:
   Goat-anti-Rabbit (1:3000 From Bio-Rad,)
   Goat-anti-Mouse (1:3000 From Bio-Rad,)
   Sheep-anti-Mouse (1:2000 Amersham Biosciences, cat# NA931V)

7. Place the membrane in a clean box, rinse with PBS-T first. Wash membrane in PBS-T. 1 X 15min, 5 X 5min.

8. Incubate membrane in a 1:1 mix of the ECL (Amersham) reagents. (~1ml of each buffer per small gel). Drain the membrane and place it on top of glass and cover

with Cling Wrap. Expose the membrane to X-ray film for seconds to minutes; you will need to try different exposure times.

*Staining the membrane in Amido black (Sigma) after western:
2 min staining in 40% methanol, 10% acetic acid, 0.1% Amido black 10B, destain in water.

*Strip the membrane and reprobe with another antibody:
wash the probed membrane in 0.1 M Glycine (pH2.4) for 10~15min ar RT, then rinse with PBS-T for 10 min twice. This does not always work.

References

Garceau NY, Liu Y, Loros JJ, Dunlap JC. Alternative initiation of translation and time-specific phosphorylation yield multiple forms of the essential clock protein FREQUENCY. Cell. 1997, 89(3): 469-76.