Western blot protocol

(Mark Hickman, February 2009)

Growth and protein extraction

1. You want ~25mL of mid-log cells (2 x 10^7 cells/ml or OD_{600}=0.67). One way is to add colony to 5mL YPD. Grow overnight at 30°C to saturation. The next morning, dilute saturated culture 1:50 and grow 4 hours at 30°C.
2. Spin cells in 50mL tube. For all the subsequent steps, keep solutions and tubes on ice. Pre-chill all tubes and centrifuges, and/or use equipment in cold room.
3. Resuspend the cell pellet in 0.5 mL cold extract buffer (add the protease inhibitors fresh; recipe below) and transfer to 2-mL screw-cap tube. Spin at max speed at 4°C in microfuge for 1 min. Remove supernatant. You can put the pellet at -80°C and perform the extraction later, or you can continue to the next step.
4. Resuspend the cells in 500 µl of extract buffer (with protease inhibitors added fresh; recipe below).
5. Add cold glass beads (acid-washed; Sigma G8772) to the tube until the level of the beads is just below the meniscus of the cell solution. Screw the cap on tight.
6. Bead Beat in cold room for 2 x 1-min pulses, with 5 minutes between pulses to keep the extracts cold.
7. Poke a hole in the top and bottom of the tubes with a 25G needle (short blue one, like ½ inch) that has been heated in a flame. Place the tube in a 5-ml Falcon tube (Falcon cat# 352054). The 2-ml tube will sit on the top of the 5-ml tube with a tight fit.
8. Spin the tubes at 1000g for 5min at 4°C, to spin the extract/cells out of the beads and into the 6-mL tube. Pipet the supernatant from the 6-mL tube (it is OK if you get some of the pellet which contains the insoluble proteins, membranes and other insoluble macromolecules) into a 1.5mL centrifuge tube.
9. Spin the extracts at max speed in a microfuge at 4°C for 10 minutes. Transfer supernatants (with soluble proteins) to a fresh 1.5 mL tube and store extract at -80°C.
10. To measure the protein concentration, dilute extract 1000-fold in water. Mix 500 µl of this diluted extract with 500 µl of Bradford reagent (also called Coomassie reagent; Pierce 1856209; take out what you need from 4°C and warm to room temp before mixing with extract). Also include a no-protein control for the blank (500µl water plus 500 µl Bradbord reagent). Mix well and leave at room temp for 5 minutes. Measure the OD_{595} with the spectrophotometer, which should be close to 0.3. This is in the linear range. You can use the standard curve that I have generated. The mg/ml of the extract is: $0.001*D*OD_{595}/0.0304$, where D is dilution (in this case, 1000).

Running gel and blotting

1. Thaw extracts, if frozen, on ice. Heat water bath or block to 70°C.
2. Prepare sample buffer: Combine ß-mercaptoethanol (FYI, undiluted stock is at 14.27M) to 10% with NuPage 4X LDS sample buffer (Invitrogen # NP0008).
3. Prepare each sample tube: First add water to bring all samples to same total volume (like 10-15 µl). Then, add sample buffer to bring to 1X in final volume. Finally, add 50-100 µg from the protein extract and mix. Place tube at 70°C for 10 minutes.
4. Put on ice for ~2 min, then spin briefly to bring all solution to the bottom of the tube.

5. Prepare NuPage gel: Make up 1L of 1X NuPage running buffer with water and the 20X mix (Invitrogen # NP0001). Take NuPage pre-cast gel (e.g., Invitrogen Mini NuPAGE Novex 10% Bis-Tris Gel 1.5mm, 10-well, cat# NP0315BOX) out of 4°C and bag. Rinse with water. Remove the white strip at the bottom of the gel. Slowly remove the well comb to avoid mis-shaping the wells (save the comb for later). With the wells facing inward, place 2 gels (or 1 gel and 1 buffer dam) against the rubber gasket of the Nupage buffer core. Put into lower buffer chamber (it only fits 1 way). Put the gel tension wedge in the buffer chamber. Fill between the gels with running buffer. If there are no leaks, fill the outside of the buffer core with running buffer.
6. Load gel: Load all the samples and 2-5 μl of the molecular weight marker (Invitrogen MagicMark XP # LC5602, peroxidase-labeled) It is useful to also include a pre-stained marker to follow electrophoresis and protein transfer (e.g., Invitrogen BenchMark Prestained Protein Ladder cat# 10748-010). Be sure to load so that you know the order of the samples (I prefer to load the marker in lane 1, my sample 1 in lane 2, and so on). Place the lid on the buffer chamber.
7. Run gel: at 200V for about 1 hr until dye reaches bottom of gel.
8. In the meantime, prepare for the transfer step by making the cold transfer buffer (500 mL is sufficient; see recipe below) and wetting the blotting materials: Specifically, put the membrane (Invitrolon PVDF, Invitrogen cat# LC2005) in a container slightly larger, and cover with 100% methanol. You can pour the methanol back into a tube for later use. Add blotting buffer to the membrane, enough so that you can add the filter paper and blotting pads. Leave at 4°C until you are ready for the transfer. (Label the membrane with a pencil. I put a designating letter in the top right corner of the membrane on the side that will contain the proteins.)
9. At the end of the run, pour out the running buffer and wash apparatus with water.
10. Take out the 2 gels. For each gel, separate the two plastic plates with the rounded edge of the comb that you obtained when preparing the gel for running (step 5 above). The gel adheres randomly to one plate so be careful to keep the adhering plate on the bottom to prevent breaking the gel.
11. Assemble the blotting sandwich. Look at the picture in the Invitrogen NuPage manual. Add materials in this order: the bottom part of the blot module, 2 blotting pads, then a filter paper. Pick up the gel with your gloved hands and place on top of the filter paper. Add blotting buffer on top of gel. Place the membrane on top and spread out the bubbles between the gel and membrane (the rounded side of the comb works well for this). Add one filter paper, one pad, then another filter paper, the other gel, some blotting buffer, another membrane (remove bubbles), a filter paper, and two more pads. Place top part of the blotting module on this “sandwich”. Put the blot module into the buffer chamber. Put the gel tension wedge in the buffer chamber. Fill the blotting module with blotting buffer to just above the gels. Fill the buffer chamber outside the blotting module with water (used to keep the blotting module cool).
12. Put on the chamber cover and move to cold room. Connect to power supply. Run at 30V for ~4 hours or overnight.
13. When finished blotting, remove the membrane carefully and put in TBS-T (recipe below) to equilibrate. In the meantime wash all the blotting components with water and allow to dry.
14. Make up primary antibody solution (recipe below).

15. Put the blot into a 96-well-size plate (with no wells, but with a cover). Add 10 mL of the primary antibody solution. (Don't let the blot dry out so add solutions immediately. Some people recommend to do a blocking step with the 5% milk solution for 1 hour but this is not necessary).
16. Incubate on a rocker at 4°C overnight for best results. (There is much controversy on these conditions, but this gives consistently good results.)
17. Wash 2 times quickly with 10ml TBS-T. Then do 3 x 5 min washes with TBS-T at room temp on rocker.
18. Make up secondary antibody solution. The antibodies from Jackson ImmunoResearch give consistently good results at a 10,000-fold dilution: goat anti-rabbit-HRP (cat# 111-035-003), goat anti-mouse-HRP (cat# 115-035-003), donkey anti-goat-HRP (cat# 705-035-003).
19. Add 10 mL of the secondary antibody solution.
20. Incubate for 1 hour on a rocker at room temperature.
21. Wash 2 times quickly with 10ml TBS-T. Then do 3 x 5 min washes with TBS-T at room temp on rocker.
22. In the meantime, prepare the ECL+ detection reagent (Amersham/GE RPN2132) using 1ml solution A and 25 μl solution B for each blot. Vortex.
23. When finished washing, hold the corner of the membrane with forceps and allow the TBS-T to drip off and then touch the lowest corner to a Kimwipe. Place the membrane protein-side-up on saran wrap.
24. Immediately add the ECL+ solution and spread over entire surface of membrane by lifting up edges of saran wrap.
25. 1 min after addition of ECL+, hold the corner of the membrane with forceps and allow the ECL+ to drip off and then touch the lowest corner to a Kimwipe. Place the membrane protein-side-down on fresh saran wrap. Stretch out the saran wrap and press on the membrane to get rid of air bubbles. Wrap the membrane with the edges of the saran wrap on the non-protein side (leaving a consistent single layer of saran wrap on the protein-side). Tape into a film cassette with the protein-side-up.
26. In the dark room, add film to the membrane and close the cassette. Make sure not to move the film, to have the bands exposed to just one area of the film. Do different exposures, starting with 20 seconds. 1 sec to 5 minutes is the range that can be used. Develop film in automatic film developer.
27. If using the membrane again, keep at 4°C wrapped in saran wrap. Right before using again, equilibrate in TBS-T at room temperature. (There are stripping protocols that are used to strip the antibodies from the membrane but this usually results in poor subsequent Westerns.)

Extract buffer (Workman; 100 mL)

4 mL 1 M HEPES pH 7.4 (40mM)
7 mL 5 M Sodium chloride (350mM)
1 mL 10% NP40 (0.1%)
25 mL 40% Glycerol (10%)
63 mL dH_2O

- protease inhibitors added fresh (Roche tablets, cat#11836170001, kept at -20°C, add 1 tablet to 10 ml of buffer)
- phosphatase inhibitors added fresh (Halt phosphatase inhibitor cocktail, Thermo cat# 78420, kept at -20°C, add 0.1 mL per 10 ml buffer)

Transfer buffer

1. Use concentrated buffer: 20X NuPage buffer (Invitrogen #NP0006) or 25X Tris-Glycine made according to Invitrogen blotting protocol (18.2 g Tris base + 90.0 g glycine up to 500 mL with water; filter sterilize and store at room temp for up to 6 months)
2. Bring up to 20% methanol, 1X transfer buffer, in water. Keep at 4°C until use, within a couple days.

TBS-T

10 ml of 1M Tris, pH 8.0 (10 mM)
30 ml of 5M NaCl (150 mM)
1 ml of Tween 20 (0.1%) (Tween is viscous. A 1-ml syringe works well for taking up and dispensing 1 ml)
up to 1L with dH_2O
Keep at room temperature for a few days.

Antibody solution

1. Add 2.5g of Carnation Instant Nonfat Dry Milk to 50ml tube and bring up to 50 ml total with TBS-T. Put on rocker at room temp for ~1 hour to sufficiently mix the 5% milk solution.
2. Take the desired amount of 5% milk solution (10ml per blot) and add to a separate tube.
3. Add the antibody (usually 1000-fold dilution, but depends on antibody; determine the best concentration empirically, by starting with the concentration in the antibody manual).
4. Keep the rest of the 5% milk solution at 4°C for less than a day.