Western Blotting of Proteins Part II

Last week we separated head extracts and blotted them. Today we will detect the presence of SNAP-25 and SNAP-24 on the western blots.

As described in last week’s handout, detecting the presence of proteins on the western blot involves 3 basic steps:

1) **Incubation with primary antibody** - this antibody is raised against the protein you want to detect (in this case SNAP-24 and SNAP-25). It is generated by injecting an animal (mouse, rat, rabbit, goat, chicken, or guinea pig) with the protein or portion of the protein you want to detect (antigen). The serum from this animal contains a high concentration of IgG against your protein of interest. This IgG will stick to the blot where the protein of interest migrated.

2) **Incubation with secondary antibody** - this antibody is raised against the IgG (usually) of the primary antibody. The antibody we are using against SNAP-24/25 was raised in guinea pig. So the secondary antibody we will use is referred to as “donkey anti-guinea pig”. In addition this antibody is chemically coupled to the enzyme horseradish peroxidase (HRP). This antibody will attach specifically to where the primary antibody is found on the blot.

3) **Detection of band** with the HRP substrate, luminol, a chemiluminescent substrate. Where ever HRP is found, the substrate will be broken down and emit light. This is detected on Xray film. Since the secondary antibody is specifically bound to the area where our protein is, it will be detected as a dark band on film.

**Things to note:**
The process of western blot detection involves blocking the membrane with a lot of other proteins so the antibodies stick only where we want them. That is why we blocked the whole blot in nonfat dry milk. Then for each antibody step we include bovine serum albumin (BSA) to prevent the antibody from sticking to the blot nonspecifically (it also prevents the antibody from sticking to the plastic dish etc). After each antibody step there is extensive washing so only well-attached antibodies remain on the blot. It is also important to figure out the appropriate dilutions of primary and secondary antibody that detects a band. Too dilute and you see nothing, too much and the blot will be a solid black rectangle.

**General procedure**
The blot will be cut into two pieces. One part will be probed with an antibody raised against the middle of SNAP-25 (which is identical with SNAP-24). The other part of the blot will be probed with an antibody against the N-terminus of SNAP-25.

The portion of the blot being probed by the SNAP-25 specific antibody (N-terminal) will establish that the deficiency removes SNAP-25. The other blot will establish that SNAP-
24 levels increase in the heat shock line and that SNAP-25ts mutation reduces the antiserum’s effectiveness, since the epitope the antibody recognizes is mutated.

Procedure:

1) Rinse blot 3 times with PBS-Tween solution to get rid of the milk solution. With a razor blade, carefully cut the blot so that each blot has a set of molecular weight markers and notch the upper left corner of each blot. Transfer one of the blots to a separate container. Make sure that a layer of PBS-tween is covering the blots at all times.
2) Drain PBS-Tween from each blot then add immediately the diluted anti-N-terminal SNAP-25 to the one blot (control, 1-16, SNAP-25ts) and the anti- SNAP-25/24 antibody to the other blot.
3) Place on the shaker for 60 minutes. Then rinse 3 times with PBS-Tween quickly. Add fresh PBS-Tween then shake 10 minutes, repeat twice more for a total of 3, 10 minute washes.
4) Add anti-rabbit horseradish peroxidase solution to the anti-SNAP-25 probed blot and anti-guinea pig horseradish peroxidase solution to the SNAP24/25 probed blot. Shake 1 hour.
5) Rinse blots 3 times quickly with PBS-Tween, then 3 times 10 minutes.
6) Cover an old piece of xray film with saran wrap and tape it on the back. Place both blots next to each other to make it whole again. Pipet 0.4 mls of Reagent 1 into a 1.5 ml tube. Pipet 0.4 mls of Reagent 2 the same tube, mix. The combination of these reagents is the substrate for the horseradish peroxidase. Pipet the solution over the whole blot and let sit 1 minute. Then drain off excess with a kimwipe. Cover with another layer of saran wrap. Place Glo-glo stickers around the blot and put into a film cassette.
7) In the dark room expose glo-glo stickers to light, then shut off lights and put on safe light. Remove a ½ piece of film from the box (the film is in a black plastic bag in the box with the opening directed down) and place over blot without moving it much. Close cassette and expose for 1 minute. Fold over bag of film and put back into box, seal box up. After 1 minute, open cassette, remove film and feed film into X-Omat be placing film against edge of slot and slowly push it till it grips the film, then let go. Wait until you hear a beep and then you can turn on the lights. Wait for film to come out of slot then orient film with the Glo-glo stickers. Mark the size of molecular weight markers.