9.12 Western Blotting 2: Detection **11.20.2006**

Note: You will start from step 2 today.

Protocol

1. Block non-specific binding sites by immersing the membrane in 5% non-fat dried milk, 0.1% (v/v) Tween 20 in Tris-Buffered Saline solution (TBS-T) for 1 hour at room temperature on an orbital shaker.
2. Put two membranes in two separate boxes and mark them GFP or CaMKII. Add 10 ml of TBS-T each. Add 2 ul of the primary anti-GFP or anti-CaMKII antibody.
3. Incubate the membrane in diluted primary antibody for 30 min at room temperature on an orbital shaker.
4. Briefly rinse the membrane with two changes of wash buffer (~20 ml) and then wash the membrane in ~20 ml of wash buffer for 5 minutes at room temperature twice.
5. Add 10 ml of TBS-T each. Add 2 ul of the secondary anti-rabbit IgG labeled with horse radish peroxidase (HRP).
6. Incubate the membrane in the diluted secondary antibody for 30 min at room temperature on an orbital shaker.
7. Briefly rinse the membrane with two changes of wash buffer and then wash the membrane in 20 ml of wash buffer for 5 minutes at room temperature twice.
8. While washing, prepare follows.
   2 ml solution A + 50 µl solution B
   Two SaranWrap on flat surface
   Paper towel
   Forceps
   Timer
9. Drain the excess wash buffer from the washed membranes and place **protein side up** on a sheet of SaranWrap.
10. Pipette each 1 ml of the mixed detection reagent on to the membrane to cover all membrane.
11. Incubate for 5 minutes at room temperature.
12. Drain off excess detection reagent by holding the membrane gently in forceps and touching the edge against a tissue. Place the blots protein side down on to a fresh piece of SaranWrap, wrap up the blots and gently smooth out any air bubbles.
13. Place the wrapped blots, protein side up, in an x-ray film cassette.
14. Place a sheet of autoradiography film on top of the membrane. Close the cassette and expose for 15 seconds.
15. Remove the film and replace with a second sheet of unexposed film. Develop the first piece of film immediately, and on the basis of its appearance estimate how long to continue the exposure of the second piece of film. Second exposures can vary from 1 minute to 1 hour.

Background information about chemiluminescent detection

Chemiluminescent detection utilizes the enzymatic generation of an acridinium ester, which

produces a light emission. Combined HRP and peroxide (usually H_2O_2) catalyzed oxidation of the Lumigen PS-3 Acridan substrate generates thousands of acridinium ester intermediates per minute. These intermediates react with peroxide to produce a chemiluminescence. The resulting light is detected on autoradiography film.

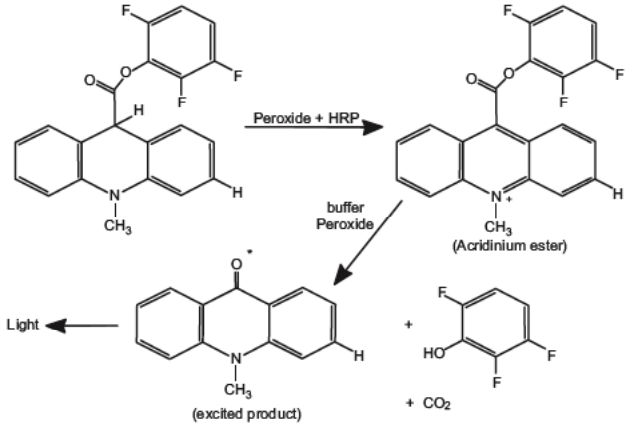

Figure 1. Chemiluminescent reaction of Lumigen PS-3 with horseradish peroxidase

Points of study and report

How does western blotting work?

Is your result as expected? If there is any problem, please describe and think about possible troubleshooting.

Destaining of CBB stained gel

1. Pour staining solution to the sink. Rince 2-3 times with tap water.
2. Add destaining solution ~20 ml
   30% methanol
   10% acetate
3. Add tissue paper in the container.
4. Float at 45 °C.
5. Check destaining occasional. Replace tissue paper occasionally.