Appendix D

Secretion Lab IV: Probing Southern Blots and Yeast Phenotypic Analysis

Reread the introduction to the first *Secretion* lab to remind yourself of the "big picture" and read about Southern blots, pp. 300–303, in Alberts *et al*., 3rd edition.

You should come to lab knowing which of your three plasmids is the vector, YEp24. **I am** *circle one:* ***positive reasonably certain*** **that plasmid ________________ is YEp24. This is because** ____.

You should come to lab with a hypothesis about which of your plasmids is RSB203 and which is RSB204. **I believe that plasmid ________________ is RSB203. This is because** ____.

You should also come to lab with hypotheses about what yeast strains will be growing on which kinds of media. This will be hard, and will require a lot of thought, but you can do it! You may want to fill this chart out with one color of pen and then use another color of pen in lab to record actual observations.

	SC-U, 25 C	SC-U, 30 C	SC-U, 38 C	SC-H-U, 25 C	SC-H-U, 30 C	SC-H-U, 38 C
Wild-type + vector						
Wild-type + 203						
Wild-type + 204						
sec-Defective (ER import) + vector						
sec-Defective (ER import) + 203						
sec-Defective (ER import) + 204						
sec-Defective (ER = >Golgi) + vector						
sec-Defective (ER = >Golgi) + 203						
sec-Defective (ER = >Golgi) + 204						
sec-Defective (Golgi = >plasma membrane) + vector						
sec-defective (Golgi = >plasma membrane) + 203						
sec-defective (Golgi = >plasma membrane) + 204						

This week we will hybridize the nylon membranes containing your DNA with a piece of DNA specific for the α-factor signal sequence region. The piece of DNA containing the α-factor signal sequence region is called a "probe." I will have labeled the probe DNA with the enzyme horseradish peroxidase. To do this, the DNA was first denatured so that it was in single-stranded form. Horseradish peroxidase (HRP), complexed with a positively charged polymer, was then added. Because of the charge

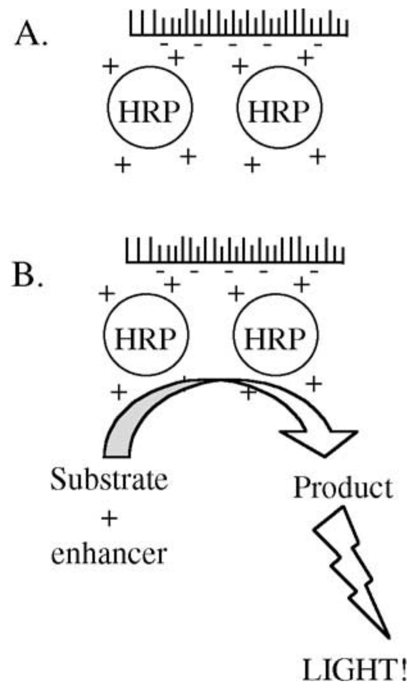

Figure 2.

interaction with the DNA backbone, the peroxidase formed a loose attachment to the nucleic acid. Addition of glutaraldehyde then caused the formation of chemical cross-links so that the probe is covalently labeled with enzyme (Figure 2).

You will use the labeled probe in hybridization with the target DNA immobilized on your membrane. From this stage onward it is important to take care that the peroxidase enzyme activity is not lost and therefore that the temperature of the hybridization mixture never exceeds 42°C (*why do you think this is important?)*. After hybridization the membrane will be washed to remove unlabeled probe, again with care taken to keep the temperature at or below 42°C. The washed filters will then be incubated briefly with the ECL detection reagents. Detection reagent 1 decays to hydrogen peroxide, the substrate for peroxidase. Reduction of hydrogen peroxide by the enzyme is coupled to the light-producing reaction by detection reagent 2. This contains luminol, which on oxidation produces blue light. The light output is increased and prolonged by the presence of an enhancer so that it can be detected on a blue-light-sensitive film (Figure 2).

PROTOCOL

Once solutions are added to the membrane, it is important that the membrane not dry out.

- Wearing gloves, retrieve your membrane and put it inside one of the plastic tubes, initialed side (DNA-bearing) toward the center of the tube. Touch the membrane as little as possible.
- Obtain an aliquot of the hybridization buffer from the incubator, and pour the buffer into the tube with your membrane, trying not to make bubbles.
- Replace the cap and roll the tube until the membrane is completely wet with buffer.
- Incubate the tube in the hybridization oven for 15 min. This step is known as prehybridization. *What do you think happens during this step?*
- After the 15 min prehybridization, add the labeled probe *into* the buffer. Do not to squirt it directly onto the membrane.
- Return the tube to the oven for 1 hr to allow the probe to hybridize to the blot.

Use this time to analyze your yeast strains.

- After the 1-hr hybridization is over, pour 200 ml of primary wash buffer into your Tupperware wash box. Retrieve the tube with your membrane and using forceps, remove the membrane from the tube, bringing along as little hybridization buffer as possible, and place the membrane into the wash box. Make certain that the lid is on tightly, and shake it in the water bath at 40.5°C for 15 min.
- During this wash, obtain the detection reagents, a small piece of bench paper, and a piece of plastic wrap.

Carefully read this last section over before proceeding because the next several steps need to be done quickly. Make sure that you understand all of these steps before the 15-min primary wash step ends. If you have questions, ask! It is OK for the primary wash to go up to 30-min if necessary.

- Pour the primary wash buffer into the sink and immediately add 20 ml of secondary wash buffer to your membrane. Shake to cover the membrane.
- Pour the 20 ml of secondary wash buffer into the sink, and add 100 ml of secondary wash buffer. Shake at room temperature on your bench for 5 min.

It is critically important that the membrane not remain in secondary wash buffer for longer than 30 min—if it does, it may strip off all the probe.

- When the secondary wash is over, use the forceps to remove the membrane from the wash buffer, and put it **initialed (DNA-bearing) side up** onto a paper towel for **30 sec** to soak up excess wash buffer. Then, transfer the membrane, DNA side up, onto the plastic side of a small sheet of bench paper.
- Pour one of the two tubes of detection reagents into the second tube. Invert a few times to mix, and then pour the mixture on top of the membrane, forming a puddle. Move the bench paper gently in a circular motion to keep the membrane completely covered with the reagents, letting them remain on the membrane for only 1 min.
- When the minute ends, lift one corner of the membrane with the forceps so the excess reagents drain off. Then, place the membrane with the **initialed (DNA-bearing) side down** on top of the plastic wrap.
- Fold the sides of the plastic wrap around the membrane, cut off the excess wrap with scissors, and gently press to remove air. It is important that the outside of the plastic wrap be dry. Blot it with a Kimwipe and seal the plastic wrap with tape if necessary.
- We will then label your membrane with fluorescent tape and expose it to film in the dark room for 15 min. We will give you the film of this exposure before you leave lab. If you need a longer exposure, we will expose it for a few hours and give you the film from that exposure later that evening or the following day.

Phenotypic Analysis of Transformants

Examine your plates and analyze your yeast transformants. Does the growth of the wild-type strain match your predictions? Why or why not? How about the growth of the *sec* mutants?