

**Experiment 6: Western Blot**

**DAY 1:**

Solutions Needed:

Gel Running Solutions (SDS sample solution, Electrophoresis Buffer, etc.)

Transfer Buffer = add 7.25g Tris-base and 33.5g glycine to 2L H<sub>2</sub>O and pH to 8.0.

Then add 600ml methanol and bring to total volume of 3L.

Dilution Buffer = 1x PBS

Blocking Buffer = 0.25ml Tween-20, 5% Carnation Nonfat Dry Milk (25g), 50ml

10x PBS, 450ml H<sub>2</sub>O. (MAKE FRESH, KEEP

REFRIGERATED.) Makes 500ml.

Note: PBS = Phosphate Buffer Saline.

**Gel Electrophoresis:**

Run 10% acrylamide gel of AP,  $\beta$ -galactosidase, cell lysate, and molecular weight marker according to the Exp 5.1 protocol.

- Use 12 $\mu$ L of AP and 21 $\mu$ L of cell lysate, and 5 $\mu$ L of Molecular Wt. Marker.

- Gels need to be loaded symmetrically:

Mkr , Lysate , Lysate , AP , AP , Mkr , Lysate , Lysate , AP , AP

- Run gels at 120V until dye line is through the stacking gel, then increase to 150V and run until dye line is less than 1/4 inch from the bottom of the gel.

**Nitrocellulose Transfer:**

Transfer proteins from acrylamide gel to nitrocellulose paper. WEAR GLOVES AT ALL TIMES, TOUCHING NITROCELLULOSE AS LITTLE AS POSSIBLE.

- Prepare the transfer box:

Add ice tray and medium size stir bar into box and fill 1/2 full with transfer buffer.

- Making the gel-nitrocellulose sandwich:

Lay out transfer cassette holder, and build from black side of holder:

1. Soak white fiber pad in transfer buffer, letting the pad absorb the buffer, lay white fiber pad on the holder.

2. Soak full piece of thick filter paper, letting paper absorb transfer buffer, then lay filter paper on top of the wet white fiber pad. Be sure there are no air bubbles.

3. Lay gel down on top of the thick filter paper. Carefully push out any air bubbles between filter paper and gel.

4. Using tweezers, pull out a piece of nitrocellulose paper (will be sandwiched between two blue sheets), mark upper left hand corner of paper for identification later (using a dull pencil, place a small mark/arrow so orientation of the paper can be recognized later) Soak the paper in transfer buffer. Then carefully lay the nitrocellulose paper down on top of gel. BE SURE PAPER LINES UP WITH GEL EXACTLY THE FIRST TIME.

Again make sure there are no air bubbles preventing the proteins from transferring over to the nitrocellulose.

5. Soak a second full sheet of thick filter paper in transfer buffer, and lay on top of the nitrocellulose. Remove all air bubbles.

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6. Soak second white fiber pad in transfer buffer and lay on top of filter paper.
  7. Close and lock cassette. Place into transfer box with clear side (also the side the nitrocellulose is on) towards the red electrode and match black with black (gel should be on this side). If nitrocellulose is not orientated to the red side and the gel with the black, turn sandwich round.
- Once sandwiches are in transfer box, add the ice filled cassette, then fill box with transfer buffer, so that the cassettes are completely submerged. Set stirrer for low speed and run transfer at **75V** for **90 minutes**.

When transfer is complete, pour transfer buffer back into the 4 L bottle and saved. Break down sandwich.

Separate the nitrocellulose membrane from the gel and place in Ponceau S stain. Often one will stain the SDS-PAGE gel in Coomassie blue solution, however for two obvious reasons we will not do this procedure. (Why do you think this step might be necessary? What two methods do we employ so we do NOT have to stain the gel?). After the bands can be visualized in the Ponceau S stain, rinse the membrane off well with DI water. Cut in half the membrane in half between the two groups of bands. One half of the membrane can be saved for a reference, the other half placed in a Petri plate to which you will add Blocking buffer (5% milk powder in 1x PBS). The membrane will remain in the blocking buffer on a rocking plate until Thursday's class.

### Day 2

#### Solutions Needed:

- Wash Buffer = 0.05% Tween-20 (0.5ml of Tween-20), 1x PBS. Makes 1L.
- Primary Antibody Solution = anti-AP conjugated to horse radish peroxidase (anti-AP-HRP) in Blocking Solution. Makes 100ml.
- 4-CN Solution: this is a substrate solution for HRP that produces a colored band on the nitrocellulose paper.

After the nitrocellulose membrane has been blocked, you will be doing the binding assay and color development:

1. We will directly add ~75ml of anti-AP-HRP conjugate solution (1:250 dilution of 1mg/ml conjugate (400 $\mu$ L) to the Blocking Buffer. The Petri plate must be covered with aluminum foil immediately because ambient fluorescent light will inactivate Horse Radish Peroxidase (HRP). Place on rocker for 90 minutes.
2. Empty out anti-AP-HRP solution, and refill with ~50ml of Wash Buffer (enough to allow the nitrocellulose to slosh around) and place on rocker for 10 minutes.
3. Empty out Wash Buffer and repeat 2 more times (total rinse 3x for total of 30 minutes.)
4. Empty out third Wash Buffer, then add ~30ml of 4-CN solution to which we will add H<sub>2</sub>O<sub>2</sub>. Cross your fingers, swirl or rock until bands develop.

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Once bands develop, pour out 4-CN Solution. Use a pen/pencil to circle the bands.

### How well did the transfer go?:

Why do you think researchers often place the SDS-PAGE gels in Coomassie staining solution following the transfer?

What two things did we do that made it possible to not have to stain the gel?

Does the number of Ponceau-S bands equal the number of 4-CN bands?

To those who have never done Westerns, the end of this experiment seems rather anti-climatic. In fact, sometimes we will hear someone say “Is that it?”. To those who have done Westerns is the realization that any successful Western is a major accomplishment. In order to gain a better appreciation of the complexities of this experiment you might want to compose a list of all the things that could go wrong and how you would try to isolate the problem if you saw no bands at the end of the experiment.