Preparation for the laboratory: Web tutorial – SDS/PAGE Western Blots

Last week you grew bacteria and induced expression of the β-galactosidase gene by adding IPTG, a lactose analog, to the cultures. You collected samples of the bacteria and indirectly measured expression of the β gene at the level of enzyme activity by quantifying the rate of conversion of ONPG to ONP.

Gene expression can also be assessed by looking directly at the relative quantity of the gene product, β-galactosidase protein. This week, we will examine the expression of the β-galactosidase protein using a technique called the Western Blot.

For this experiment, aliquots of E. coli cells were sampled by Darcy Blankenhorn at the same time points following IPTG addition you used last week and prepared for electrophoresis. This week, proteins in these E. coli lysates will be separated on polyacrylamide gels and blotted to a nitrocellulose membrane (“the blot”). Next week, we will probe the blot with an anti-β-galactosidase antibody to assess the relative quantity of the protein.

Background: SDS-PAGE (PolyAcrylamide Gel Electrophoresis):

Chemical conditions: SDS (sodium dodecylsulfate) is an anionic detergent that both disrupts the 2⁻ and 3⁻ structures of proteins and coats them in negative charges. Running protein gels in the presence of SDS therefore equalizes the charge on the proteins and linearizes them for passage through the gel matrix. Thus, proteins will run through the gel in proportion to only their molecular weight, not their diameter, shape, or charge:mass ratio. The loading buffer also contains a reducing agent, β-mercaptoethanol, which disrupts protein structure by breaking disulfide bonds between cysteine residues.

Bacterial lysates were prepared for electrophoresis by mixing cell pellets with gel loading buffer. The loading buffer will make the sample sink to the bottom of the wells in the gel. It also contains a stain that will make it possible to track the movement of the proteins. The polyacrylamide gel you will use was prepared in the BioRad factory. The first task for you is to load your sample on the gel. Work in pairs.

Preparation of the Electrophoresis apparatus:

The instructor will demonstrate how to assemble the electrophoresis apparatus (Fig. 1) in class. A brief description follows here.

1. To prepare the gel cassette, remove the comb from the top, exposing the wells. Carefully pull the tab at the bottom of the cassette, removing a small portion of adhesive cover and exposing the gel through the slot in the gel plates.
2. To assemble the apparatus, place the gel cassette into the electrode assembly. Be sure the small plastic protrusions at the top of the cassette align properly with the green gasket on the electrode assembly. The shorter side of the gel cassette should face inward. Each electrode assembly will accommodate two gel cassettes.

3. Place the electrode assembly and gel cassettes into the clamping frame. At this point, the swinging cams should be pointed outward, roughly perpendicular to the gel cassette.

4. Push down on the electrode assembly while closing the cam levers of the clamping frame. Each cassette should form a tight seal with the green gasket.

5. Lower the entire assembly into the buffer tank and add enough room temperature running buffer to the inner tank to just cover the wells. You are now ready to load your gels.

Thaw your pellet 2 minutes at 95°C and make sure that the pellet is resuspended. Work the pellet up and down using a pipett to resuspend it. **Use care to avoid making bubbles.**

After you assemble your gel, load your samples in the wells in the following order:
- 10 µl Molecular weight markers;
- 20µl of each of the following lysates (1); (3); (4); (5); (6); (7). These numbers correspond to the treatments used in the Lac Operon lab and represent the control for the role of IPTG (1). Tubes 3-7 correspond to the timing of chloramphenicol addition 0, 15, 30, 45 and 60 minutes respectively.

Before running the gel check the level of the running buffer. It should be about a ½ inch above the top of the wells. Run the gel at 200 V (60mA) until the blue dye reaches the bottom of the gel (approx. 45 minutes to 1 hour).

While the gel is running prepare for the western blot. After electrophoresis is complete, turnoff the power supply and disconnect the electrical leads. Remove your gel from the electrophoresis assembly.
Preparation for Western Blotting:

Like electrophoresis, western blotting uses electrical current to move proteins. In this case, the proteins are driven from the polyacrylamide gel onto an adjacent membrane.

**While your gel is running, prepare the blotting apparatus.**

1. Fill the ice chamber with ice and place it in the freezer.
2. Fill a Pyrex dish with cold western transfer buffer.

Once the gel run is finished, place the clamping frame in the large plastic box, remove the gel cassette and discard the used buffer.

**The following steps are done in the Pyrex dish.**

When opening the cassette case determine to which side of the case the gel is attached. Place that side on the bottom of the Pyrex dish. Carefully open the cassette so the gel is on the lower side. Cover the gel with a wet filter paper. Flip the plate over and place the filter paper on the pad that is resting on the black cassette. Remove the gel cassette and continue building the sandwich underneath transfer buffer so the sequence is as follows:

black cassette wall → scotchbright pad → filter paper → gel → nitrocellulose membrane → filter paper → scotchbright pad → red or clear cassette wall.

Use care to avoid bubbles between the gel and the membrane.

**Make sure that the gel is closer to the black cassette wall and the membrane is closer to the red wall.** The current runs toward red and the samples move onto the nitrocellulose membrane.

Place the cassette inside the BioRad Transfer Blot Cell along with the ice pack or place in a mouse cage filled with ice. Place a small stir bar in the cell. Cover the cassette completely with cold transfer buffer from the Pyrex dish. The electrodes should be in the center of the apparatus so that they will fit properly in the lid. Attach the lid and place the apparatus on a stir plate and initiate stirring.

Run the blot at 100 V for one hour.

Remove the blot and place it in about ~ 15 ml of Ponceau Red stain for 5 minutes. Pour the stain back into the bottle and rinse the blot with deionized water until the background disappears. Scan an image of the stained blot, if desired. This indicates the efficiency of protein transfer.
Place the blot on a piece of filter paper. Allow it to air-dry (~ 5 min), and wrap it in Saran Wrap. Wrap again in foil; label; store blot at 4° until next week.

24 hours before next week’s lab: Report to 109 to place your blot in blocking solution. This will only take a few minutes but is critical. Remove your blot from the refrigerator. Place it in the plastic container provided. Add 20 ml of blocking solution. Wrap the plastic dish in parafilm and place the blot on the rocker table at room temperature until tomorrow. Check with your instructor if you have questions.

Next week in class, we will probe the blots with 1° antibody, wash them, probe them with 2° antibody, wash them again, and use detection reagents to develop the signal. Review this concept using the schematic in Appendix 1 or the web tutorial.

Report to lab 1 hour before the normal scheduled time - after setting up the blot you can take a 1 hour break. See details under procedures for week 2.

Development of Western Blots – Week 2

This is the week when we determine the relative induction of β-galactosidase protein expression by IPTG. We do so by developing the western blot that you performed last week.

Review of experiment to date: Previously, you grew bacteria and sampled the culture before and after the addition of IPTG, a lactose analog that should induce β-galactosidase expression. Last week, you subjected these samples to SDS-PAGE and blotted the gel to a nitrocellulose membrane. This week, we will probe the membrane with antibodies in order to detect the expressed protein.

Basic overview of immunoblot detection of proteins: Specific detection of proteins on the western blot is based on the use of two antibodies (Ab's)—[1] a 1° antibody specific for the target protein, and [2] a 2° antibody that binds the 1° antibody. Importantly, an enzyme, alkaline phosphatase, is covalently bound to the secondary antibody. This allows detection of a specific protein by a sort of domino effect: the 1° Ab binds the protein on the blot; the enzyme-conjugated 2° Ab binds the 1° Ab; and the complex is detected by incubating the blot with colorimetric enzyme substrates. The alkaline phosphatase catalyzes a reaction that turns a soluble substrate into a blue precipitate on the blot. Thus, the presence of a blue band on the blot corresponds to the presence of the target protein. The identity of the band is further confirmed by its size (116 kDa), indicated by its mobility in the gel.

Source of reagents.

In our experiment, the 1° Ab specifically binds the β-galactosidase protein. This Ab was produced by injecting a rabbit with protein (the "antigen.") The rabbit's immune system, recognizing the injected protein as foreign (the β-galactosidase was purified from *E. coli* bacteria), produced antibodies against it. These antibodies are soluble proteins that circulate in the blood. Blood serum drawn from the injected rabbit contains these antibodies. The
antibody we will use to probe our western blots was derived from this serum. We did not need to perform this process ourselves. The anti-β-galactosidase antibody is marketed by eBioscience, Inc.

The 2\textsuperscript{nd} Ab was similarly produced in a goat by injecting it with rabbit antibodies. One end of an antibody protein (the “constant domain”) is the same for all antibodies in a species, while the other (the “variable domain”) is specific to a certain antigen. The goat’s immune system, recognizing the constant portion of the rabbit antibodies as a foreign protein, made antibodies against that. In other words, the rabbit antibody was the antigen for the goat antibody. [Say that three times quickly]. This 2\textsuperscript{nd} antibody can bind to any antibody produced by a rabbit immune system. It is termed a goat anti-rabbit antibody.

The goat anti-rabbit antibody is cross-linked to the enzyme alkaline phosphatase. This means that the enzyme is covalently bound to the antibody. The presence of the enzyme does not interfere with the antibody's ability to bind its target antigen. The enzyme is crucial for detecting the antibody because it catalyzes a reaction with color-changing substrates, as described above. The alkaline phosphatase-conjugated 2\textsuperscript{nd} Ab is also a commercial product obtained from Sigma Chemical Company. We call it by the acronym “GARAP” (goat anti-rabbit alkaline phosphatase). In the presence of BCIP/NBT the alkaline phosphatase turns blue.

**Specific Protocol.**

Your membranes have been in blocking solution for 24 hours. This is a crucial step in preparing for a Western Blot. Blocking solution is 10% non-fat dry milk dissolved in phosphate-buffered saline (PBS) and 0.3% Tween-20 (a mild detergent). It provides a high concentration of proteins that will bind all over the nitrocellulose membrane. Saturating the membrane with a mixture of generic proteins will prevent later non-specific binding by our 1\textsuperscript{st} and 2\textsuperscript{nd} antibodies.

**Running a Western Blot:**

1. **On your scheduled lab day, report to lab 1 hour prior to the start of class.** Pour off the blocking solution. Add 10 ml blocking solution containing 50 µl of 1\textsuperscript{st} Ab to your blot. This can be found in the refrigerator. Close the dish and incubate for one hour with constant shaking on the rocker table.

2. **At normal lab start time:** Collect the Ab/blocking solution in a capped tube and indicate with a mark that it has been used. Rinse the blot briefly with ~20 ml of wash buffer (1X PBS with 0.3% Tween-20). Repeat. Now wash the blot with ~20 ml of wash buffer for 10 minutes on the rocker table. Discard this wash buffer and repeat the final step.

3. In a capped tube make 2\textsuperscript{nd} Ab/blocking solution by mixing 10 ml of blocking solution with 10 µl of 2\textsuperscript{nd} antibody. Close the dish and incubate for one hour.

4. Discard 2\textsuperscript{nd} Ab solution and rinse/wash the blot as described in step 2.
5. a) Rinse the blot briefly in ~20 ml of AP buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂). Discard.
   b) Add 10 ml of BCIP/NBT solution to the blot. Place the blot back on the shaker. Over a few seconds or minutes (this may take 20 minutes), the light purple to blue bands should appear before your eyes.
   c) When the blot is sufficiently developed, pour off the solution and incubate the blot with a stop solution (20 mM Tris-HCl, pH 7.5, 5 mM EDTA). This provides a pH unfavorable to the alkaline phosphatase activity and chelates the Mg²⁺ ions that the enzyme requires, thus stopping the reaction. Dry the blot in air.

6. ASAP: Use a scanner to capture an electronic image of your blot. Save this image in .tiff format to your H: drive. You will open the image in Adobe Photoshop, crop it, paste it into PowerPoint, and prepare a labeled figure. See Appendix 2 on how to prepare figures.

Assignment:
Incorporate the results of the western blot experiment into a synthetic paper that includes the results of the β-galactosidase and the lac operon experiments. The paper should focus on two major themes, the activity of β-galactosidase and the genetic regulation of β-galactosidase synthesis. You examined the effect of temperature and pH on β-galactosidase activity then explored regulation using two techniques; an ONPG assay and a western blot. This paper should use the format from Biology 109 and include a title, introduction, methods, results, discussion, acknowledgments, and references.

Introduction: briefly develop the background that leads to the hypotheses for each experiment.
Methods: explain the experimental design for the β-galactosidase activity experiment. Refer to the lab manual for detailed techniques for the other experiments.
Results: subset the results to reflect the experiments. Be sure to include a carefully labeled image of your gel and in the text point out the major features of the gel.
Discussion: this section should be divided into two sections, one on the activity of the enzyme and one that focuses on regulation. The following questions may help guide your thoughts.
   1. Why are both pH and temperature important regulators of enzyme activity. Are the effects of one more dramatic than those of the other?
   2. Is there evidence that the timing of chloramphenicol addition affects the amount of enzyme present? Is this pattern consistent with your predictions?
   3. Is IPTG necessary for the production of the enzyme? How might you explain the presence of the enzyme without IPTG induction?
   4. Are the results of the ONPG assay for β-galactosidase production consistent with those of the western blot? Try to explain any differences.

Your paper is due the week after Spring Break

Acknowledgments:
We would like to thank Darcy Blankenhorn for assisting with the technical development of this laboratory.

Preparation for next week: Tutorial 4 - PCR