Detection of β-galactosidase Expression

You may recall from earlier in the semester that β-galactosidase is a bacterial enzyme that hydrolyzes polysaccharides which are linked together by β-galactosidic bonds. The colorless substrate o-nitrophenol-β-galactoside (ONPG) can be hydrolyzed by this enzyme to yield galactose and the colored compound o-nitrophenolate (ONP). ONP is a bright yellow compound, the presence of which is easy to assay using a spectrophotometer.

This week you will measure the β-galactosidase activity of the yeast you transformed with the PDR5-lacZ reporter construct. The level of enzyme activity in your cells serves as a read-out of transcription activity. In addition to assaying samples of yeast carrying your mutant allele, you will analyze the control strains prepared by your instructor. The positive control is a pdr3-11 strain transformed with the PDR5-lacZ construct. The negative control is a wild-type strain transformed with the PDR5-lacZ construct. Your mutant yeast should be compared to these two controls.

This assay is a little tricky and involved, so you will first perform the assay with one sample of the pdr3-11 control, so you can get the hang of things before tackling the remaining samples. This will also help you devise a system so that you and your partner can work quickly and efficiently. Once you feel comfortable with the assay, label all the tubes you'll need, and go for it!

Permeabilized Cell Assay
adapted from Kaiser et al. (1994)

1. **One day before your lab period**, start three 1-ml cultures from three different colonies of your mutant transformed with the PDRE-β-gal plasmid. The cells should be grown in the small, capped tubes containing SDC-ura. The lack of uracil will force the strain to maintain the PDR5-lacZ reporter construct. Put these cells into the 30°C shaking water bath.

2. **Four hours before your lab period** add 2ml of fresh SDC-ura to your cultures, briefly vortex each tube to resuspend any cells that have settled to the bottom, then return them to the shaking water bath.

3. At the beginning of lab, get a tube of the pdr3-11 (positive control) culture out of the shaking water bath. Briefly vortex this culture to resuspend any cells that have settled to the bottom of the tube. Measure the absorbance of this sample at 630nm with the Spec20 labeled “red”, using 3ml of SDC-ura as your blank (See page 71 if you need a refresher on how to use a Spec20). Record this value in your lab notebook.
5. Transfer 1ml of the culture to a microfuge tube.
6. Pellet the cells by centrifuging for 1 minute at 13,000rpm in a microcentrifuge.
7. Use a pipetman to remove as much media as possible, without disturbing the cell pellet.
8. Now go to the chemical hood and resuspend your sample in 1.0 ml of Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM 2-mercaptoethanol) and transfer this cell suspension to a small glass test tube. Add one additional ml of Z buffer to the glass tube. At this point you should also start preparing your blank which contains everything but cells. So go ahead and add 2ml of Z buffer to an empty tube.
9. Add 6 drops of chloroform and 4 drops of 0.1% SDS to each tube (your sample and your blank), then cap the tubes and return to your lab bench.
10. Vortex the mixtures at top speed for 10 seconds and then incubate the samples in a 28°C water bath for 5 minutes. This incubation will permeabilize the cells. (Note: because the substrate has not been added yet, your sample should NOT be turning yellow. If it is changing colors, see your instructor for assistance!)
11. Return to the chemical hood and start the reaction by adding 0.4 ml of 4 mg/ml ONPG in Z buffer to each tube (including your blank) and vortex briefly (less than 1 second) to mix. **Start your timer!!!** (Note: now your sample should start turning yellow)
12. Incubate the tubes at 28°C until the mixture has acquired a pale yellow color.
13. Stop the reaction by adding 1.0 ml of 1.0M Na$_2$CO$_3$, leaving the tube uncapped as briefly as possible. Of course, the blank should also get 1.0 ml of 1.0M Na$_2$CO$_3$, because it is supposed to contain everything except cells! Gently shake tubes to mix. **Record the precise amount of time elapsed since you added ONPG.**
14. Remove cap and use an appropriately blanked Spec20 labeled “blue” to measure the absorbance of the reaction at 420nm. Record this value in your notebook, and replace the cap on your tube.
15. Calculate the activity in β-gal units using the following formula

\[
\frac{1000 \times A_{420}}{A_{630} \text{ of assayed culture} \times \text{volume assayed (ml)} \times \text{time (minutes)}}
\]

- $A_{420}$ is the reading you got from step 14.
- $A_{630}$ is the number you got from step 4.

*The “volume assayed” is 1ml, because that is the amount of yeast culture you used.*

15. Repeat this procedure for two more samples of *pdr3-11* yeast, three samples of wild-type yeast, and three samples of your mutant yeast. To keep from getting overwhelmed, you should conduct these experiments with only 2-3 samples at a time. **Note: you do not need to prepare any additional blanks.** The one you made up will suffice for the remainder of the lab period.
17. After acquiring all your data, construct a graph for incorporation into both your notebook and your final lab paper. The data should be presented as a bar graph, where each bar represents the average β-gal units for the three samples of a particular strain of yeast. These bars should also have error bars which reflect standard deviation. This graph should be made in SigmaPlot. Directions for how to create this graph are provided below.

**Constructing a Bar Graph for Your Transactivation Assay Results**

- Open SigmaPlot
- Enter the data for the wild-type strain in column 1
- Double click on the heading for column 1 and enter the name “wild-type.”
- Enter the data for the positive control (pdr3-11) in column 2
- Double click on the heading for column 1 and enter the name “pdr3-11.”
- Enter the data for your mutant in column 3
- Double click on the heading for column 1 and enter the name “HePC mutant.”
- At the top of the page, click on “Graph,” then “Create Graph.”
- From the list of graph types, select “Vertical Bar Chart,” then click “Next.”
- From the list of graph styles, select “Simple Error Bars,” then click “Next.”
- From the list of symbol values, select “Column Means,” confirm the error calculations are set to “Standard Deviation,” then click “Next.”
- Under data format select “Many Y,” then click “Next.”
- Under data for bar 1, select “wild-type.”
- Under data for bar 2, select “pdr3-11.”
- Under data for bar 3, select “HePC mutant,” then click “Finish.”
- Delete the title and legend from the graph.
- Double click on “Y Data,” replace it with “beta-galactosidase units,” and click “OK.”
- Double click on “X Data,” and replace it with “wild-type  pdr3-11  HePC” (Note that there should be ~12 spaces between each strain name!)
- Delete the numbers and the ticks along the X axis.
- The graph is now ready to be cut and paste into your paper!
How to use a Spectronic 20 colorimeter

1. Turn the instrument on by rotating the power knob in a clockwise direction. Allow about 10 minutes for the instrument to warm up.
2. Use the wavelength control knob to set the instrument to the desired wavelength.
3. With the sample holder empty and closed, turn the left hand knob (zero control) until the meter needle reads 0 on the Percent Transmittance scale. This is called zeroing, because no light is being transmitted.
4. Into the sample compartment, insert a cuvette that contains all of the substances in the solution except the light absorbing compound you wish to measure. This is called the blank. Close the cover.
   
   **Cuvettes should be clean, dry, and free of fingerprints and scratches!!!**
5. Rotate the right hand light control knob until the absorbance scale reads zero.
6. Without touching either dial, put the unknown samples in the tube holder and read the absorbance on the dial. The needle should return to zero when the tube is removed. Check the calibration occasionally with the blank, and adjust if necessary.
7. Remember to turn off the machine when you are through using it.