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5.36 Biochemistry Laboratory
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SESSION 2 (lab open 1-5 pm)

1.) Expression of the H396P Abl kinase domain in the presence of Yop phosphatase

Inoculate 500 mL of LB/ kan/ strep with your 10-mL starter culture of bacteria containing the ABL and YOP genes. Place the flask in the 37 °C shaker and periodically check the OD600 (optical density or absorbance at 600 nm) using the UV/Vis spectrometer. Use (non-inoculated) LB as the sample blank. Review the spectrometer instructions in Appendix A2. Once the OD600 reaches 0.1, the density should double approximately every 20 minutes, which should help you plan when next to check the OD. When the media reaches an OD600 of 0.8 to 1.0, remove the flask from the 37 °C shaker and place it in the 24 °C shaker. At this point, transfer 1 mL of the culture to a 1.5-mL eppendorf tube and spin it down for 3 minutes in a bench top microcentrifuge. Remove the supernatant and store the pellet at -20 °C. This is your pre-induction sample for subsequent gel analysis. After the cell culture has been shaking at 24 °C for at least 10 minutes (or up to one hour), induce protein expression with the addition of IPTG. Add 100 µL of 1M IPTG to give a final concentration of 0.2 mM IPTG. Let the culture grow at 24 °C overnight.

2.) Isolation of the wt Abl plasmid DNA from the two 6-mL overnight cultures.

While you monitor the OD of your H396P Abl(229-511)/ Yop expression, you should concurrently work to isolate the plasmid DNA from your two 6-mL overnight bacterial cultures, which contain the wt Abl(229-511)-encoding plasmid. You will use the isolated plasmid DNA in later lab sessions for DNA mutagenesis to create a Bcr-Abl mutant implicated in Gleevec-resistant CML.

Today you will use a Qiagen miniprep kit to isolate the plasmid DNA. The following instructions are modified from the Qiagen miniprep handbook:

Miniprep Procedure:

- a.) Harvest the bacterial cells from your overnight cultures (12 mL total) by transferring 1.5 mL of culture into each of four 1.5 mL eppendorf tubes and spinning down the cells in a microcentrifuge for 3 minutes. Discard the supernatant and add 1.5 mL of remaining culture to the four eppendorf tubes. Repeat the centrifugation and discard the supernatant. You should have a small bacterial pellet at the bottom of each tube.
- b.) Check that RNaseA has been added to Buffer P1. Add 250 µL of Buffer P1 to each cell pellet and completely resuspend each pellet by vortexing.
- c.) Add 250 µL of Buffer P2 to each tube, and mix by inverting the tubes 4-6 times. Do not vortex, since that can cause shearing of the DNA. If you have properly lysed the cells, the cell suspensions will turn blue after the addition of the P2 buffer. If there are colorless regions or brown clumps in the cells, continue mixing until a homogenous blue solution appears.
- d.) Add 350 µL of Buffer N3 to each tube and mix immediately by inverting the tubes 4-6 times. The solutions should become colorless and cloudy.

- e.) Centrifuge the tubes for 10 min at 13,000 rpm in you bench top microcentrifuge. A compact white pellet should form in each sample.
- f.) Apply the resulting supernatant from each tube (which contains the plasmid DNA) to each of four QIAprep spin columns by decanting or pipetting.
- g.) Centrifuge for 30-60 s. Discard the flow through.
- h.) Wash the spin columns by adding 0.75 mL Buffer PE and centrifuge for 30-60 s. Discard the flow through.
- i.) Centrifuge for an additional 1 min to remove residual wash buffer. This is essential for the success of any future enzymatic reactions.
- j.) Place the QIAprep spin columns into clean and labeled 1.5-mL microcentrifuge tubes. To elute DNA, add 40 μ L of Buffer EB warmed to 55 °C (10 mM TrisCl, pH 8.5) to the center of each spin column, let stand for 1 min, and centrifuge for 1 min.

Combine the DNA elution samples (160 μ L total) in an eppendorf tube. For long-term storage, keep the purified DNA, labeled with your names and the date, at -20 °C.

3.) Quantification of the DNA concentration by absorption at 260 nm

Dissolve x μ L (suggested amount is 3 μ L) of DNA sample in 100 μ L of water. Measure the absorbance at 260 nm in a quartz cuvette. See Section Appendix A2 for UV/Vis spectrophotometer instructions. To calculate the concentration in μ g/ μ L of your double stranded DNA, multiply the Abs₂₆₀ by (0.05)(100/x). Typical concentrations of miniprepped DNA are 0.1-2 μ g/ μ L.

SESSION 2B (lab open 1-2 pm)

Preparation and storage of the H396P Abl(229-511)/Yop- containing cell pellet.

- a.) Remove the 1-L culture flask from the shaker and transfer the contents into a 500-mL centrifuge tube.
- b.) Prior to spinning down the culture, transfer 1 mL from the centrifuge tube into a 1.5-mL eppendorf tube and spin it down for 3 minutes in a bench-top microcentrifuge. Remove the supernatant and store the tiny pellet at -20 °C. This is your post-induction sample for subsequent gel analysis.
- c.) Balance the mass of your centrifuge tube with a blank or with another group's tube. If needed, use a transfer pipette to adjust the amount of culture in each tube or to add buffer.
- d.) Spin down the cell culture in the centrifuge at 6K rpm for 10 minutes at 4 °C. Make sure the centrifuge is balanced, and, when possible, combine your centrifuge run with that of other groups to minimize wait time.
- e.) As the culture is spinning down, tare a 50-mL conical tube and record the weight on the tube and in your notebook.

f.) Once the centrifuge run is complete, decant the supernatant and carefully remove any residual liquid with a transfer pipette. To discard, treat the supernatant with 50% bleach, and then pour it down the drain with plenty of water. **The protein is in the pellet.**

g.) Carefully scrape the pellets into the tared 50-mL conical tube using a spatula.

h.) If needed (if the pellet is not transferring well from the 500 to the 50-mL tube), add some buffer to transfer any remaining pellet stuck in the 500-mL tube. Spin down the pellet in the 50-mL conical tube for 5 minutes using the centrifuge tube adaptors and making sure your sample is balanced with a blank. Discard the supernatant.

i.) Weigh the tube and record the pellet weight.

j.) Store the H396P Abl(229-511)/Yop-containing cell pellet in the -20 °C freezer until needed for protein purification. Please make sure to write your names and the date on your tube!