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5.36 Biochemistry Laboratory
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SESSION 10 (lab open 1 to 5 pm)

1.) DpnI digestion of the PCR reaction

As described in the Quikchange mutation overview (in Session 3), the purpose of DpnI treatment is to digest away the template (wild type) DNA, such that the only remaining plasmid is the mutation-containing PCR product. Sufficiently high DpnI concentration and digestion time are essential for complete digestion of the template DNA in order to prevent carrying over the wt plasmid into the transformation, which could result in the isolation of wt, instead of mutated, DNA. For each 50- μ L PCR reaction, transfer 10 μ L of the crude reaction into a fresh tube. Add 1 μ L of DpnI (10 units/ μ L) and mix thoroughly by pipetting 4-6 times. Transfer the 10- μ L reaction to a new tube to ensure that all the DNA in the tube has been exposed to DpnI. Incubate the digestion at 37 °C for at least 1 hour. The crude digestion product can be used directly in a transformation reaction. Extra PCR and digestion products should be labeled and stored at -20 °C.

2.) Preparation of LB/kan agar plates

While the DpnI digestion incubates, prepare LB/kan agar plates for your transformation reaction. To prepare 4 plates, weigh out 5 g of LB powder and 3 g of bacto-agar in a 200-mL flask. Add 100-mL of water, and cover the top of the flask with aluminum foil. Add a piece of autoclave-indicator tape to the side of the flask and autoclave the solution for 20 min on the liquid cycle. Several groups should autoclave their media in a single autoclave run to avoid extra waiting time. Once autoclaved, allow the solution to cool to approximately 55 °C, such that you can hold the flask comfortably. Add 100 μ L of 1000X kan to give a final concentration of 50 μ g/ mL, and swirl the flask to mix. Immediately transfer 25-mL aliquots of the mixture to cell culture plates using a 100-mL pipette. Allow the agar to solidify for at least 15 minutes before using. Store any unused plates at 4 °C.

3.) Transformation of DH5 α cells

Bacterial transformation involves the uptake of foreign DNA into a cell for subsequent expression. While a small percent of bacterial species (approximately 1%) have the genetic capacity to take in outside DNA through the cell membrane, transformation is commonly achieved using procedures that render bacterial cells passively permeable by heat shock or electroporation. In this procedure, you will use heat-shock competent cells, which work best with plasmid DNA, for your transformation. Heat-shock competent cells are prepared by incubating the chilled cells with divalent metal ions to increase their permeability. After incubation with plasmid DNA, briefly heating the cells results in uptake of the foreign plasmid. Careful timing is essential to maximize DNA incorporation while minimizing cell destruction.

Plates should be placed in the 37 °C incubator with the LB/agar side up.

- I. For each transformation reaction, thaw a single 30- μ L aliquot of heat-shock competent DH5 α cells in an ice bucket. Today you will set up a total of four transformation reactions: three for the mutagenesis products and one as a positive control with your isolated wt plasmid DNA. Competent cells cannot be refrozen for later use, so please do not remove more aliquots than you will use from the -80 °C freezer.
- II. Keep your plasmid DNA from the DpnI digestions and your miniprep of wt Abl DNA chilled in the ice bucket
- III. For each transformation reaction, add 25 μ L of cells and 1 μ L of DNA (either from the crude DpnI digestion mixture or from your miniprep) in a 1.5-mL sterile eppendorf tube on ice. Typically, the DNA samples used are 0.5 to 1 μ g/ μ L. Mix the cells gently by stirring with the pipette tip.
- IV. Heat shock the cells by placing the tubes in a 42 °C water bath for exactly 45 seconds. Use a timer to ensure that the cells are not overexposed.
- V. Immediately transfer the tubes to an ice bucket. Incubate the cells on ice for 2 minutes, then add 500 μ L of SOB to rescue the cells.
- VI. Transfer the cell solutions to cell culture tubes.
- VII. Incubate the tubes at 37 °C in a shaker (220 rpm) for 30-60 minutes. 30 min. should be adequate for routine transformations, such as this one.
- VIII. For each cell solution, plate 200 μ L onto a room temperature or 37 °C LB/kan agar plate that has been divided into 4 sections by marker on the bottom of the plate. Streak the plate as shown by your TA across the four quadrants. Label each plate as mutant or wt, specify the point mutation on the mutant plates, and include the amount of DNA template used for the mutant reactions. Write your name and your TA's name on each plate as well.
- IX. Incubate the plates overnight in the 37 °C incubator.

SESSION 10B (lab open from 1-2 pm)

Pick individual colonies to grow up for DNA isolation (miniprep)

Add 3 mL of autoclaved LB solution (from your 100-mL preparation in week 1) into each of 3 cell culture tubes. Confirm that your LB media is contamination-free by checking that the solution is clear. Add 3 μ L of a 1000X (50 mg/ mL) kan stock solution to each tube to give a final concentration of 50 μ L/mg kan.

Remove your LB-kan agar plates from the 37 °C incubator. Confirm that your wt Abl transformation was successful in generating hundreds or thousands of colonies on the designated plate. Estimate and record the number of colonies on the plates transformed with DNA from your mutagenesis with 10, 20, or 50 ng of template DNA.

Using a sterile pipette tip, transfer a colony to a cell culture tube from the mutant DNA plate with bacteria transformed with the mutagenesis mixture using the least amount of template DNA. For example, if you have colonies on all of your plates, use the plate from the mutagenesis reaction with 10 ng of DNA. This further decreases the likelihood of selecting a colony that contains wt DNA.

Select 2 more colonies from the same mutant DNA plate, and inoculate the solutions in the other 2 cell culture tubes. Be careful to never mix colonies, since each colony contains a different DNA clone and mixing could result in combining wt DNA with DNA containing the desired mutation.

Place the culture tubes in a 37 °C shaker overnight. On Wednesday you will isolate the plasmid DNA from the 3 selected colonies.

For any groups that experimented with a new point mutation (that hasn't been optimized for this lab), you may or may not have colonies from your mutant DNA transformations. If you are not in one of the lucky groups with colonies, you should pick a colony from another group's plate and record your new target mutation. Quikchange mutagenesis can require optimization and does not work for every mutation imaginable, and your grade will NOT be affected by whether or not your mutagenesis succeeded, assuming your primer design and Quikchange procedure were competently executed.