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5.36 Biochemistry Laboratory  
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## SESSION 9 (lab open 1-3 pm)

During laboratory Sessions 9 and 10 you will perform site-directed mutagenesis to construct the DNA for a mutant Abl kinase domain with a single amino acid substitution. You will transform cells for subsequent isolation of your mutant DNA.

### 1.) Preparation of mutagenesis primers

For the mutagenesis reactions 10  $\mu\text{M}$  solutions of your forward and reverse primers should be prepared with ddH<sub>2</sub>O. Before opening the primer containers, which contain your primer DNA as lyophilized powder, briefly centrifuge the tubes for 30 to 60 seconds. Calculate and add the necessary amount of water to achieve a 10  $\mu\text{M}$  solution, and store your primer solutions at 4 °C short term and at -20 °C long term.

### 2.) Amplification of the mutant plasmid by PCR

You will set up a series of PCR reactions with various concentrations of DNA template (your isolated plasmid DNA) to produce plasmids containing the desired base-pair substitution. By varying the amount of DNA template, you will determine the reaction conditions that ultimately produce colonies with the least amount of template DNA possible, which maximizes the likelihood of isolating a colony with the desired mutation. While there must be enough template DNA to facilitate replication, too much template can result in incomplete digestion after the PCR, resulting in undesired amplification of the wild type DNA. Also essential for successful mutagenesis is the use of PfuTurbo as the DNA polymerase. Unlike standard PCR reactions, which require replication of a 1 to 2 kb insert, mutagenesis requires replication of an entire plasmid, which can be 5 or more kb (the pEt plasmid is approximately 6.1 kb with the Abl insert). PfuTurbo is used for its high fidelity and ability to extend very long templates.

In each of three sterile PCR tubes, add the following components in the order listed, where  $x$  = the volume of miniprep elution added (in  $\mu\text{L}$ ) to give 10 ng of DNA.

	<b>10 ng DNA</b>	<b>20 ng DNA</b>	<b>50 ng DNA</b>
10X Pfu buffer	5 $\mu\text{L}$	5 $\mu\text{L}$	5 $\mu\text{L}$
template plasmid DNA	$x$ $\mu\text{L}$	$2x$ $\mu\text{L}$	$5x$ $\mu\text{L}$
10 $\mu\text{M}$ forward primer	1.2 $\mu\text{L}$	1.2 $\mu\text{L}$	1.2 $\mu\text{L}$
10 $\mu\text{M}$ reverse primer	1.2 $\mu\text{L}$	1.2 $\mu\text{L}$	1.2 $\mu\text{L}$
10 mM dNTP mix	1.0 $\mu\text{L}$	1.0 $\mu\text{L}$	1.0 $\mu\text{L}$
ddH <sub>2</sub> O	$40.6 - x$ $\mu\text{L}$	$40.6 - 2x$ $\mu\text{L}$	$40.6 - 5x$ $\mu\text{L}$
Immediately before starting the PCR machine, add:			
PfuTurbo, 2.5 units/ $\mu\text{L}$	1.0 $\mu\text{L}$	1.0 $\mu\text{L}$	1.0 $\mu\text{L}$
Final volume	<hr/> 50 $\mu\text{L}$	<hr/> 50 $\mu\text{L}$	<hr/> 50 $\mu\text{L}$

Mix the reactions gently by pipetting 4-6 times, and place the tubes in the thermal cycler. The length of the PCR program is critical for successful mutagenesis because the Pfu

polymerase requires ample time to extend around the full plasmid. Your TA will demonstrate how to set the following PCR program: 95 °C for 30 sec; [16-20 cycles of 95 °C for 30 sec: 55 °C for 1 min: 65 °C for 2 min per kb plasmid]; then hold at 10 °C. Since the plasmid you are using is approximately 6 kb, each 65 °C extension will be for 12 min. The class should load the PCR reactions in the thermal cycler together and the TA will start the run. The run will take over 4 hours, and the reaction tubes may be left overnight in the cycler after the run is complete.

**SESSION 9B** (lab open from 1 to 2)

- 1.) Remove your reaction from the thermal cycle and store at 4 °C.