WEEK 3: Plasmid DNA Minipreps

In WEEK 1, we performed a LIGATION REACTION to insert a 750 bp portion of the tobacco hornworm cation transporter cDNA into the pGEX-2T plasmid. In WEEK 2, we used the ligation reactions to TRANSFORM E. coli cells (DH5α strain), inserting our newly-engineered plasmid into the bacteria. Successfully transformed bacteria cells give rise to colonies on LB-ampicillin plates.

In WEEK 3 we will grow up several of the colonies in liquid culture and then purify the plasmid DNA that each contains. We will assess the structure of the purified plasmid by digesting it with restriction enzymes EcoRI and Xho I—the same enzymes used to prepare the sticky ends of the plasmid vector and DNA insert. Digestion of the new plasmid with these two enzymes should result in the production of two linear DNAs identical to the ones we initially joined—~750 bp and ~ 4.9 kb. In short, we put the plasmid together in the ligation reaction; now that we have replicated many times in the living bacteria, we want to take it apart again to make sure the parts are still the same. If everything checks out, then we can proceed with expression of our engineered protein encoded by the plasmid.

Plasmid mini-prep procedure.

Day 1: Wednesday, Jan 31. Report to the lab at 3:00 PM to start your cultures.

1. Adding antibiotic to the bacterial growth medium. LB medium is pre-made for your convenience. Ampicillin stock solution is 50 mg/ml. How much ampicillin stock should we add to 25 ml LB to make the final concentration 100 µg/ml? __________ µl or __________ ml Add the amount to the LB in a 50-ml plastic centrifuge tube (a "falcon tube").

2. Using sterile technique (instructor will demonstrate), pipet 2 ml of LB-amp into each of 10 sterile culture tubes. Label each tube clearly with a number (1-10), initials, and date.

3. Using a sterile toothpick, "pick" a colony from one of your transformation plates. Using sterile technique, place the toothpick into a culture tube. Repeat for the other nine tubes. Place the inoculated cultures into the shaking incubator and grow them overnight at 37˚C and 225 rpm shaking. Why 37˚C? What is the purpose of shaking the tubes?

Day 2: Thursday Feb. 1 (normal lab meeting time).

A. Breaking open the cells.

1. Fill 1.5 ml flip-cap butes with culture. Place the remaining culture in the refrigerator for later use.

2. Spin the tubes for 1 minute in the microfuge. Decant the medium using a P1000 or a vacuum line with pasteur pipette.

3. Mix TES buffer with lysozyme: 3 ml TES buffer (pre-made) plus 240 µl lysozyme (10 mg/ml stock). What is the purpose of these two reagents?

   Completely resuspend the pellets by vortexing.
4. Boil tube in water bath for 1 minute in a boiling rack. [Why boil? what does the rack do?] Remove tubes from rack. Spin them for 15 minutes at full speed in the microfuge.

5. Remove bacterial debris ("the snot pellet") from each tube using a sterile toothpick.

B. Recovering the plasmid DNA.

6. Add 125 µl 7.5M NH₄OAc; mix; add 400 µl 100% isopropanol; mix.

7. Let tubes site 5 minutes at room temperature; spin 5 minutes in microfuge. Carefully remove the supernatant

8. Add 500 µl 70% ethanol; spin 5 minutes in microfuge. Carefully remove ALL supernatant. A "drawn-out" pasteur pipette works well for this. I will demonstrate how to heat the pipette in the flame and pull it into a fine-tipped instrument. Air dry the pellet for ~10 minutes. Dissolve the pellet in 40 µl of TE buffer.

C. Restriction Enzyme Digestion to check for insert presence and size.

1. Prepare a master mix according to the following table:

<table>
<thead>
<tr>
<th>1X</th>
<th>ingredient</th>
<th>11X</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.0 µl</td>
<td>H₂O</td>
<td>143.0 µl</td>
</tr>
<tr>
<td>1.0 µl</td>
<td>RNase A (10 mg/ml)</td>
<td>11.0 µl</td>
</tr>
<tr>
<td>2.0 µl</td>
<td>10X Buffer H (Promega)</td>
<td>22.0 µl</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>EcoRI Enzyme (12 U/µl)</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>0.5 µl</td>
<td>XhoI Enzyme (10 U/µl)</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>18.0 µl</td>
<td>Final volume</td>
<td>198.0 µl</td>
</tr>
</tbody>
</table>

Mix well by pipetting the mix up and down; avoid bubbles. Split the mix ten ways, 18.0 µl each (there will be 18.0 µl left over).

You now have 10 reaction tubes and 10 miniprep DNAs. A 2 µl of a DNA sample to a tube so that each reaction contains a different DNA. Mix by pipetting, as above.

Incubate the reactions at 37˚ for at least 1 hour in a heat block. Add 4.0 µl of 6X gel load buffer, mix, and freeze reactions (-20˚) until next week.

Next week, we will run our restriction digestions on agarose gels. During the Thursday laboratory, you will mix your gel, pour it, run your samples, and prepare the electronic image.