APh 162- Molecular Biology

Day 3

Qiagen PCR purification of Double Digested YFP PCR product
Perform as on Day 1.

Nanodrop reading of purified, double digested YFP PCR product
See TA demo, as on Day 1.

Ligation of Vector and Insert
This step will incorporate our gene of interest into the open, linear vector we created during the double digest.

Reagent:	**Reaction:**	**Control:**
Double digested backbone DNA (from pZE21_LacZ)*	**50 ng**	**50 ng**
Insert DNA	**Calc.**	**0 μl**
Quick Ligase Reaction Buffer (2X)	**10 μl**	**10 μl**
Quick Ligase	**1 μl**	**1 μl**
DDH$_2$O	**Calc.**	**Calc.**
Total Volume	**21 μl**	**21 μl**

* Your TA will provide the concentration.

Procedure:

1. To maximize production of vector + insert ligated product, we add the insert in molar excess.
    a. **Calculate the amount (mass and corresponding volume) of insert DNA required to have a <u>1:3 *molar* ratio of vector to insert</u>.**
        i. Vector length - ~2200 bp
        ii. Insert length - ~720 bp
2. To a microcentrifuge tube, add the appropriate amount of DNA and record the total volume. If the volume is below 10μl, add DDH$_2$O to bring the volume to 10 μl.
3. Add 10 μl of Quick Ligase Reaction Buffer and mix well.
4. Ask your TA to add 1 μl of the Quick Ligase, which contains an optimized ligation enzyme.
5. Incubate at room temperature for 5 mins. Then put the reaction on ice, until **Cell Transformation** (see below).
6. Perform a no-insert control by repeating these steps and omitting the insert – in its place add an appropriate amount of DDH$_2$O.
   What does this control test for?

PCR Purification of Ligated Product
Perform as on Day 1. Purify both the ligated product and the control.

Cell Transformation
A culture of the *E. coli* strain DH5α (plasmid-free) was previously made competent, that is, able to accept a plasmid, following a protocol for electrocompetent cells.

Note: Make sure your ligated DNA has been purified before this step. See the included “PCR Purification Protocol” from Qiagen.

Procedure:

1. Warm up 1 mL LB media per transformation in a culture tube at 37°C.
2. Put two electroporation cuvettes and two vials of electrocompetent cells on ice.
    a. Allow the tube of cells to thaw on ice.
1. Pipette 2 μl (~8ng) of ligated DNA into the tube containing the cells and mix gently.
2. Pipette 50 μl of this mixture into the electroporation cuvette, and electroporate at 1.8 kV. The time constant (tau value) should be 3-4 msec.
3. Immediately pipette 750 μl of warm LB from the culture tube into the cuvette, mix gently, and put back into the culture tube.
4. Incubate culture tube with cap loose on shaker at ~225 rpm for 1 hour at 37°C.
5. Plate culture on LB + Kanamycin + X-Gal plates
6. Let the plate dry, then incubate it upside-down overnight at 37°C.
7. With two separate transformations, make sure to transform cells with both the ligated product and the no-insert control.