I. INTRODUCTION:
Today you will transform *E. coli* to glow yellow by inserting a plasmid with the gene for YFP (yellow fluorescent protein). To do this, we will perform four steps: prepare the insert by copying the YFP insert by PCR, remove the *LacZ* gene from the backbone with restriction enzyme digestion, ligate the YFP gene in its place on the backbone and transform *E. coli* via electroporation to express the genes on the plasmid. A schematic of the full procedure is shown below; however in the interest of time there are some steps the TAs have done ahead of time that you will not do, which are indicated in blue in the protocols below.
The keys to this “swap” are restriction enzymes. These enzymes cut DNA based on a recognition sequence, a region of DNA with a particular pattern of bases. For example, EcoRI cuts DNA like so:

![A diagram showing EcoRI recognition sequence](image)

This results in two “sticky ends” that can then match to DNA of the correct sequence. We will digest the backbone plasmid using two of these restriction enzymes, KpnI and HindIII, to separate the LacZ gene from the rest of the backbone. Using two different enzymes with different recognition sites and different “sticky ends” allows us to insert the YFP gene in the correct orientation. Along the way, we will explore PCR and how it duplicates DNA and how gel electrophoresis moves fragments of DNA in an agarose gel.

**Note**: In many of the protocols to come, **Reagents** will be listed as 2X or 5X or 10X, etc, meaning that (unless otherwise directed) they should be added to relevant solutions such that the **final** concentration of the reagent is 1X.
II. RESTRICTION ENZYME DIGESTION

Both the LacZ gene in the pZE21 plasmid and the new reporter gene (or “insert”) that is the product of the PCR in the next section are flanked by restriction sites for the enzyme “KpnI” on one side and “HindIII” on the other. This allows us to cut the LacZ gene out and leave precisely controlled “sticky-ends” on the plasmid DNA, onto which we will ligate our glowing insert. The enzyme KpnI cuts at any site with the sequence ...5-GGTAC’C-3... leaving the sticky-end GTAC, while the enzyme HindIII cuts at any site with the sequence ...5-A’AGCTT-3... leaving the sticky-end AGCT. The fact that these sticky ends are distinct means we can discourage the plasmid from ligating on itself, and ensure that we insert the new reporter gene in the correct orientation. A myriad of available restriction enzymes ensures that we can control each piece of the plasmid independently.

Restriction enzyme concentrations are measured in the units of “activity”, where 1 unit of enzyme can digest 1 µg of DNA in 1 hour at 37°C. To ensure that all of the DNA is cut by the enzyme, it is generally recommended to add enzyme in excess of the required amount (usually by a factor of 2 or 3).

Follow the protocol below, and mix the following reagents to begin this enzymatic reaction. Once the reaction is finished, we will examine the products on an agarose gel to determine their size and differences in topology (see section IV below). In addition to the two commercially available size-standards, or ladders, to run on the gel, we will create our own “DNA Ladder” using the distinct enzyme EcoRI to digest λ-phage (viral) genomic DNA that has already been digested once with HindIII.

**Digest of λ-phage DNA:**

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Amount:</th>
</tr>
</thead>
<tbody>
<tr>
<td>HindIII-digested λ DNA, 0.5 µg/µL (NEB-N3012)</td>
<td>1.5 µg</td>
</tr>
<tr>
<td>NEB EcoRI Buffer (10X)</td>
<td>1X</td>
</tr>
<tr>
<td>BSA (10X) (NEB-B9001S)</td>
<td>1X</td>
</tr>
<tr>
<td>EcoRI (10 units/µL) (NEB-R0101)</td>
<td>2 µL</td>
</tr>
<tr>
<td>DDH₂O</td>
<td>Calc.</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>25 µL</td>
</tr>
</tbody>
</table>

**Double Digest of Plasmid:**

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Digest</th>
<th>Control 1</th>
<th>Control 2</th>
<th>Control 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid (pZE21_LacZ ) DNA, 173 ng/µL</td>
<td>300 ng</td>
<td>300 ng</td>
<td>300 ng</td>
<td>300 ng</td>
</tr>
<tr>
<td>NEB Buffer 2 (10X) (NEB-B7002S)</td>
<td>1X</td>
<td>1X</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>BSA (10X) (NEB-B9001S)</td>
<td>1X</td>
<td>1X</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>KpnI (10 units/µL) (NEB-R0142)</td>
<td>1 µL</td>
<td>0 µL</td>
<td>1 µL</td>
<td>0 µL</td>
</tr>
<tr>
<td>HindIII (10 units/µL) (NEB-R0104)</td>
<td>1 µL</td>
<td>1 µL</td>
<td>0 µL</td>
<td>0 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>25 µL</td>
<td>25 µL</td>
<td>25 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>

*Double Digest of Insert: This step would be performed on the PCR’d insert in the next section; in the interest of time, the TAs will provide you with PCR’d, digested insert when you need it.*

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Digest:</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFP (Venus) DNA: PCR results stored on ice</td>
<td>17 µL</td>
</tr>
<tr>
<td>NEB Buffer 2 (10X) (NEB-B7002S)</td>
<td>1X</td>
</tr>
<tr>
<td>BSA (10X) (NEB-B9001S)</td>
<td>1X</td>
</tr>
<tr>
<td>KpnI (10 units/µL) (NEB-R0142)</td>
<td>1 µL</td>
</tr>
<tr>
<td>HindIII (10 units/µL) (NEB-R0104)</td>
<td>1 µL</td>
</tr>
<tr>
<td>DDH₂O</td>
<td>Calc.</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>25 µL</td>
</tr>
</tbody>
</table>
Notes:
NEB Buffer 2 is one of many potential buffers, chosen to optimize the performance of both KpnI and HindIII. See “Enzyme Properties” at:

Procedure:
1. Verify all calculated reagent volumes with your TA.
2. Keep all reagents on ice when you’re not actively pipetting into or out of them.
3. For the given concentration of plasmid DNA, calculate the volume that corresponds to 300 ng.
4. Mix this volume of DNA along with the specified amounts (see table: Dougle Digest) of buffer, restriction enzymes and water to bring the total volume to 25 µL, in a 0.65 mL eppendorf tube. Add the restriction enzymes last! (See the TAs to obtain enzyme, once all other components have been added.)
5. Spin the reagents in the micro-centrifuge for a few seconds to sediment all the contents.
6. By pipetting up and down with a P20 pipette, ensure that all the reaction’s contents are thoroughly mixed, but avoid making bubbles!
7. By repeating these steps and selectively omitting enzyme, perform two single digest controls, and one no-digest control. Make sure all reactions are 25 µL in total – this will require adjusting the water volume.

Q: What do these controls tell you?
8. In addition to using commercial ladders, we will create our own ladder using viral DNA.
   a. Repeat these steps with λ-phage DNA (see table: Digest of λ-phage DNA), specifically:
      i. The λ-phage DNA has already been digested by HindIII, and we will now digest it with the restriction enzyme EcoRI.
      ii. Notice instead of 1X NEB Buffer 2, we are using the better suited 1X EcoRI buffer.
      iii. Notice we are using 2 µL of EcoRI enzyme – there is more DNA to be cut in this reaction!
      iv. Bring total volume to 25 µL with DDH₂O.
   b. Questions:
      i. The λ-phage genome is 48,502bp, if the sequence was totally random, how many HindIII (5A’AGCTT3) and EcoRI (5G’AAATTC3) sites would you expect? How many are there in reality?
      ii. Given the actual restriction sites for EcoRI and HindIII, calculate the expected segment lengths of the DNA ladder. (See the attached restriction site map for the λ-phage genome.)
Place all five of your eppendorfs that contain the 25 µL reactions in a 37°C incubator for 2 hours. After this incubation, we are ready to run the DNA agarose gel (see section IV below). The TAs have separately done a similar digest and gel, but then have purified the band of DNA corresponding to the “backbone” from the gel and will provide this gel-purified backbone for Section V. (If you’re curious, the protocol for a gel extraction is similar to the PCR purification described in a later section.)
III. Q-PCR (QUANTITATIVE POLYMERASE CHAIN REACTION) OF THE YFP INSERT

In this step we will amplify the insert DNA, and manually remove samples to be run on an analytical gel to observe the exponential increase in insert concentration. The gene we are amplifying is called Venus and is a variant of YFP (in this protocol we refer to them interchangeably).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Reaction</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert Plasmid (pZS2LacUV5O1_Venus), 18.8 ng/µL</td>
<td>10 ng</td>
<td>0 ng</td>
</tr>
<tr>
<td>Forward Primer (10 uM)</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Reverse Primer (10 uM)</td>
<td>2.5 µL</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Phusion HF Mastermix (2X)</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>DDH₂O</td>
<td>Calc.</td>
<td>Calc.</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

*Procedure:*

1. In a **thin-walled** PCR tube, mix the DNA, primers, mastermix and water together. **Keep your tube on ice!** Clearly label the tube with your initials. Briefly centrifuge this tube to pull all liquid to the bottom. We will call this tube the “PCR reaction”.
2. Label seven tubes for each of the seven time-cycle points at which we will pause the PCR reaction to take a sample. (e.g. PCR0, PCR7, PCR14, PCR21, PCR28, PCR35, Final)
3. Take 3 µL from the PCR reaction, place it in PCR0, and put that tube on ice.
4. Hopefully the idea of thermally cycling during PCR is conceptually clear, now we must program the actual device to perform this task – your TA will help you with this.

<table>
<thead>
<tr>
<th>Step:</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - Initial DNA Denaturation</td>
<td>98°</td>
<td>45s</td>
</tr>
<tr>
<td>2 - Denaturation</td>
<td>98°</td>
<td>8s</td>
</tr>
<tr>
<td>3 - Annealing</td>
<td>62°</td>
<td>10s</td>
</tr>
<tr>
<td>4 - Extension</td>
<td>72°</td>
<td>15s</td>
</tr>
<tr>
<td>5 - Final Extension</td>
<td>72°</td>
<td>5 min</td>
</tr>
<tr>
<td>6 - Hold</td>
<td>4°</td>
<td>Indef.</td>
</tr>
</tbody>
</table>

Steps 2 – 4 will repeat a total of 35 times.
Q: How much total amplification is that? What limits maximum amplification?

5. As precisely as possible, **PAUSE** the thermocycler at the *end* of the extension step (step 4) on every 7th cycle (roughly every 8 minutes). Remove the PCR reaction, take 3ul from it, place it in the appropriate PCR# tube, and place that tube on ice. Re-insert the PCR reaction tube into the thermocycler and continue the PCR process.

6. At the very end of the PCR, take 3ul of the PCR reaction and 3ul of the Control reaction, and place them in the tubes labeled as “Final” and “Control” respectively. Keep the rest of the reactions in the original tubes, and store them on ice.

7. Each of the PCR# samples and the Control will then be run on a DNA agarose gel (see the following).
   
   Q: **Describe what you expect this gel to look like.**

**PCR Purification of the Digested Insert (done by the TAs ahead of time)**

The digested insert DNA now also contains a lot of other junk (e.g., free nucleotides) that will decrease the efficiency of ligation. Because of this, the digested result needs to be purified using a simple procedure and the QIAquick PCR purification kit from Qiagen. This protocol can be found below (since you’ll be PCR purifying your ligation products!)
IV. DNA AGAROSE GEL
The primary method employed throughout molecular biology is to subject DNA in an ultra-viscous agarose gel to an electric field, wherein we utilize the ~5.88e/nm charge on DNA to “pull” it through the gel. In a given period of time the DNA migrates through the gel roughly proportional to the inverse of its mass. The above PCR reactions have produced a range of DNA amounts, though mostly the same size. It is immensely useful to be able to measure the amount of DNA created during each cycle of the PCR. Though this is an approximation of standard QPCR, you will be able to see the fluorescence of insert band increase with the number of cycles.

To properly gauge the size of DNA segments that appear on the agarose gel, we need to add a so-called “DNA Ladder” that contains segments of known length, against which we will compare our PCR results. We will use two commercially available ladders, the 100bp and the 1kbp DNA ladder respectively.

<table>
<thead>
<tr>
<th>Reagents:</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TAE- 1% agarose gel</td>
<td>~50 mL (0.5 g agarose + 50 mL TAE)</td>
</tr>
<tr>
<td>100bp DNA Ladder (NEB-N0467S; 50 µg/mL)</td>
<td>1 µL ladder + 4 µL H₂O + 1 µL 6x loading dye</td>
</tr>
<tr>
<td>1000bp DNA Ladder (NEB-N3232; 500 µg/mL)</td>
<td>1 µL ladder + 4 µL H₂O + 1 µL 6x loading dye</td>
</tr>
<tr>
<td>For each of PCR 7, 14, 21, 28, 30, 35, final, and control</td>
<td>3 µL reaction +2 µL H₂O +1µL 6x loading dye</td>
</tr>
<tr>
<td>For plasmid digest, 3 controls, λ DNA</td>
<td>25 µL of digest + 5 µL 6x loading dye</td>
</tr>
<tr>
<td>1X TAE Buffer</td>
<td>~500 mL</td>
</tr>
</tbody>
</table>

**Note:** TAE (Tris-acetate EDTA) buffer

DNA Ladders
a) Example of 1000bp DNA ladder on a 0.8% agarose gel, stained with EtBr.
b) Example of 100bp DNA ladder on a 1.3% agarose gel, stained with EtBr.
Total amount of DNA in each gel is 0.5 µg/lane. Use this to calibrate the amount of DNA in your gel.

**Materials:**
gel box; gel power supply; gel tray; gel comb

**Procedure:** (note the TAs will have already poured gels for you; skip to step 7)
1. Using DI water, clean the gel box, gel comb and gel tray.
2. In short bursts of ~10s, heat the 1X TAE- 1% agarose gel in the microwave until it is completely liquefied.
3. Secure the gel comb in the gel tray and orient so that the red rubber seals form a liquid-tight dish. Pour ~35mL of the liquid agarose into the gel tray. Be careful not to introduce any bubbles.
4. Allow the gel to solidify over the next 30 minutes, without disturbing it.
5. Once fully solidified, carefully remove the gel comb by pulling it straight up, taking care not to damage the wells. Carefully remove the gel tray (with the gel) and rotate it 90 degrees so that the wells are closer to the negative terminal.
6. Gently pour 1X TAE buffer until the reservoirs on both sides of the gel are filled and a thin layer (~1mm) of
buffer is covering the gel. The wells / holes left by the gel comb should now be filled with 1X TAE buffer.

7. Set the gel power supply to 110 VDC and 30 minutes, but do not start it yet.
8. For each PCR tube from the QPCR section, add 1ul of 6X loading dye and 2 µL H2O (now total volume 6 µL) and mix gently with the pipette tip. To each digestion product from Section II, add 5ul of 6X loading dye (now total volume 30 µL) and mix gently with the pipette tip. The loading dye permits visualization of the lanes as the gel runs. Additionally, the added glycerol will sediment the DNA to the bottom of the lane.
9. With a steady hand, place each sample in a different lane. **Note:** Be sure to record in which lane you put which sample! We may standardize this as a class.
10. Secure the gel box lid and start the power supply. Make sure the gel is running in a flat, stable orientation.
11. Within a few minutes you should see the loading dye moving in the gel.

**Visualizing DNA in the Gel**

1. The gel power supply will automatically shut-off after 30 minutes. Pour off the excess 1X TAE buffer from the gel box, being careful to keep the gel in the gel tray.
2. Place the gel into the EtBr Bath and let it stain for 9 minutes. Recall that the bath contains the dangerous chemical EtBr. This compound uniformly intercalates in the DNA and becomes fluorescent upon binding. Hence, the EtBr fluorescence intensity is proportional to the amount of DNA present in each band.
3. Destain for 20 minutes.
4. Image the gel
   a. Put the gel on a horizontal UV lamp and image the DNA bands. Make sure to adjust the focus, position and exposure to get the best gel image possible – this may require help from a TA.
   b. **Questions:**
      i. Does the pattern of the bands appear as you expected? Describe what your gel looks like.
      ii. Knowing the concentration of DNA in the 100bp ladder, you can integrate the fluorescence in each band to determine the initial concentration of each piece. Using Matlab, estimate the concentration of DNA corresponding to each PCR time point, and quantitatively describe how the DNA gets amplified over time.
      iii. Using Matlab and the 100bp ladder, calibrate the distance of each band in your gel, and verify that you produced the correct DNA lengths.
      iv. Using Matlab and the known concentration of DNA in the 100bp ladder, estimate the initial concentration of DNA by integrating the fluorescence in each band. What are some sources of error in this estimation?
      v. How are migration distance and DNA length/mass related?
      vi. Are you missing any bands? If so, where might they be?
      vii. Did the uncut plasmid control and each of the single digest controls migrate the same distance? If so, why? If not, why not?
V. LIGATION OF VECTOR AND INSERT
This step will incorporate our gene of interest into the open, linear vector created by double digest, that TAs have provided for you.

<table>
<thead>
<tr>
<th>Reagent:</th>
<th>Ligation:</th>
<th>Control:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested Plasmid (pZE21_noLacZ) DNA, 55 ng/µL</td>
<td>50 ng</td>
<td>50 ng</td>
</tr>
<tr>
<td>Digested Insert (Venus) DNA, 24 ng/µL</td>
<td>Calc. (Molar ratio 1:3)</td>
<td>0 ng</td>
</tr>
<tr>
<td>DDH2O</td>
<td>Calc. (Bring DNA to 10ul)</td>
<td>Calc. (Bring DNA to 10ul)</td>
</tr>
<tr>
<td>Quick Ligase Reaction Buffer (2X)</td>
<td>1X</td>
<td>1X</td>
</tr>
<tr>
<td>Quick Ligase</td>
<td>1 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>22 µL</strong></td>
<td><strong>22 µL</strong></td>
</tr>
</tbody>
</table>

**Procedure:**
1. To maximize production of vector + insert ligated product, we add the insert in molar excess. Calculate the amount (mass and corresponding volume) of insert DNA required to have a **1:3 molar ratio of vector to insert**. (Vector length ~2200 bp and Insert length ~720 bp)
2. Also, perform a no-insert control by repeating these steps and omitting the insert – in its place add an appropriate amount of DDH$_2$O.
   **Q: What does this control tell you?**
3. To an eppendorf tube add the appropriate amount of DNA and record the total volume. If the volume is below 10ul, add DDH2O to bring the volume to 10 µL.
4. Add the Quick Ligase Reaction Buffer and mix well.
5. Add 1 µL of the Quick Ligase, which contains an optimized ligation enzyme and mix well.
6. Incubate at **room temperature** for 5 mins. Then put the reaction on ice.

VI. PCR PURIFICATION OF THE LIGATED PRODUCT
Purify both the ligated product and the control. This removes salts and other components of the buffer that interferes with electroporation. (Note that this step is not necessary for chemical transformations!)

1. Add 5 volumes of Buffer PB to 1 volume of the DNA sample in an eppendorf tube and mix. For example, add 500 µL of Buffer PB to 100 µL DNA sample.
2. To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 s at speed of 13,000 rpm.
3. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are reused to reduce plastic waste.
4. To wash, add 0.75 mL Buffer PE to the QIAquick column and centrifuge for 60 s at speed of 13,000 rpm.
5. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the **empty** column for an additional 1 min at speed of 13,000 rpm.
   **IMPORTANT:** Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
6. Place QIAquick column in a clean 1.5 mL microcentrifuge tube.
7. To elute DNA, carefully add 30 µL Buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge the column for 1 min at speed of 13,000 rpm.
   **IMPORTANT:** Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. **Be careful do not poke the membrane with the tip!**
8. Use the NanoDrop spectrophotometer to determine the concentration of your purified ligated product. **Please see the TA demo.**
VII. TRANSFORMATION
A culture of the *E. coli* strain DH5α was previously made electrocompetent, that is, able to accept a plasmid after an electric shock. (Cells can also be made chemically competent, meaning they take up DNA after a temperature shock.)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Ligated Product</th>
<th>No Insert Control</th>
<th>Uncut Vector Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified DNA</td>
<td>2 µL</td>
<td>2 µL</td>
<td>1 µL</td>
</tr>
<tr>
<td>Electrocompetent Cells: DH5α</td>
<td>53 µL</td>
<td>53 µL</td>
<td>53 µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>55 µL</td>
<td>55 µL</td>
<td>54 µL</td>
</tr>
</tbody>
</table>

*Procedure:*
1. Warm up 1 mL LB media per transformation in a culture tube at 37°C.
2. Put three electroporation cuvettes and three vials of electrocompetent cells on ice. Allow the tube of cells to thaw on ice.
3. Pipette 2 µL (~8 ng) of the purified DNA into the tube containing the cells and mix gently by stirring around with the tip.
4. Pipette 50 µL of this mixture into the electroporation cuvette, and electroporate at 1800 V. The time constant (τ value) should be 3-4 msec.
5. Immediately pipette 750 µL of warm LB from the culture tube into the cuvette, mix gently, and put back into the culture tube.
6. Incubate culture tube with cap loose on shaker at ~225 rpm for 1 hour at 37°C.
7. Pipette 100 µL of the culture on a LB + Kanamycin + X-Gal plate that was specialized for blue-white screening.
8. Evenly distribute the liquid culture on the plate using sterile glass beads. Please see the TAs for demo.
9. Let the plate dry, then incubate it upside-down overnight at 37°C.
10. You will need to do three separate transformations, one each of the Ligated Product, the No-Insert Control and the Uncut Vector Control. We may also have you transform with the original Venus-containing plasmid as a control for what the fluorescent cells should look like.