Cloning outline/ protocol

*This protocol applies for mutagenesis (small insertions, base changes, deletions) using the InFusion Cloning kit only

1. Run gradient PCR to linearize and amplify vector
   - Objective: to figure out which melting temperature (Tm) works best for each primer set
   - Gradient PCR: allows to use a range of Tms at the same time. One of our PCR machines can do this. I'll show you how to do it.
   - Choose a Tm range: ~ -2°C of lowest Tm & + 2°C of highest Tm
   - Using wild type vector as DNA template
   - Using specific forward and reverse primers that insert mutations of interest -> these primers are unique for each mutant
   - Using Q5 High-Fidelity DNA polymerase
   - Don't forget to calculate the appropriate elongation time. For the Q5 polymerase method this is: 30secs/kb of expected PCR product
   - After setting up the gradient PCR protocol in the PCR machine, don't forget to take a picture of and/or write down Tms included in the gradient for future reference

2. Run gradient PCR product samples in 1% agarose gel
   - Sample prep: 8ul PCR product + 2ul 6X loading dye (for a 1X final concentration)
   - Don't forget to include a ladder! Recommended ladder: 1kb Plus DNA ladder
   - Run: 1XTBE, 120V, ~30mins
   - Image gel (will show you how) & save picture in usb. Also include in notebook.

3. Large scale PCR
   - Choose the Tm that worked best for each set of primers. Don't forget to write down the chosen Tm for future reference.
   - Run a large scale PCR reaction at the chosen Tm.
   - Using wild type vector as DNA template
   - Using specific forward and reverse primers that insert mutations of interest -> these primers are unique for each mutant
   - Using Q5 High-Fidelity DNA polymerase
   - Don't forget to calculate the appropriate elongation time. For the Q5 polymerase method this is: 30secs/kb

4. PCR cleanup
   - Pull together all PCR aliquots for each mutant and do a PCR cleanup using the Clonetech kit.
   - Follow kit's protocol
   - **Elute in 20ul WATER**

5. Run PCR purified large scale PCR product samples in 1% agarose gel
   - Sample prep: all of the PCR product after PCR cleanup (~20ul) + 20ul 6X loading dye
   - Gel prep: use large well comb (to fit all the sample)
   - Don't forget to include a ladder! Recommended ladder: 1kb Plus DNA ladder
   - Run: 1XTBE, 120V, ~30mins
   - Image gel (will show you how) & save picture in usb. Also include in notebook.
   - SAVE GEL FOR NEXT STEP

6. Gel extraction
   - Extract bands of interest using the Zymo Gel Purification kit.
   - Follow kit's protocol
   - Elute in 15ul WATER

7. NanoDrop gel extraction product
   - Objective: to measure the concentration of the linearized DNA product and to confirm the purity of the sample
   - Concentration should be >20ng/ul
   - 260/280 ratio should be ~1.8
   - 260/230 ration should be ~2.0-2.2

8. InFusion reaction
   - Objective: to re-circularize plasmid
   - Follow protocol on spreadsheet and in InFusion website

9. Transformation of Stellar Competent Cells (E. coli strain)
   - Follow manufacturer's protocol (pdf sent to you)

10. Inoculation of LB cultures with colonies from transformation
    - I will teach you how to do this

11. Plasmid prep using Zymo kit
    - Objective: to extract re-circularized plasmid from bacteria
    - Follow kit's protocol
    - Elute in 30ul WATER

12. Sequencing of new mutant plasmids
    - Objective: to confirm that the mutations were inserted
    - See sequencing spreadsheet for calculations
    - Will show you how to prepare the sequencing order and drop off the samples