Blunt-end cloning

Preparing the insert:

- See the PCR protocols page for "General insert amplification with Vent." Note whether or not the primers are phosphorylated at the 5'-end.
- Analyze the PCR product on an agarose gel. Run a preparative agarose gel, excise the correct insert band, and purify using Qiagen's gel purification kit.
- Measure the DNA concentration using the Nanodrop.

Preparing the vector backbone:

- See the PCR protocols page for "Whole plasmid amplification with Phusion." **Note that the DpnI digestion step is critical.**
- After the DpnI digest, run the sample on a preparative agarose gel, excise the correct band, and purify using Qiagen's gel purification kit.
- Measure the DNA concentration using the Nanodrop.
- Make a working stock solution containing 20 ng/μL of backbone DNA in water.

Ligation with inserts amplified using 5'-phosphorylated primers:

- The ligation mix should contain around 20 ng of backbone DNA and 200 ng of insert DNA. For inserts <500 bp, use 100 ng.

10x T4 DNA ligase buffer	1 μL
backbone DNA	1 μL of 20 ng/μL stock
insert DNA	X μL to get 200 ng
T4 DNA ligase	0.5 μL
ddH_2O	to total volume of 10 μL

- Incubate at room temperature for 60 min.

Ligation with inserts amplified using non-phosphorylated primers:

- Treat 200 ng of the insert with T4 polynucleotide kinase (PNK) to phosphorylate the 5'-ends. For inserts <500 bp, use 100 ng.

10x T4 DNA ligase buffer	0.5 μL
insert DNA	X μL to get 200 ng
T4 PNK	0.5 μL
ddH_2O	to total volume of 5 μL

- Incubate at 37 °C for 30 min, then cool to room temperature.
- Add the following to the reaction mix:

ddH_2O	3 μL
10x T4 DNA ligase buffer	0.5 μL
backbone DNA	1 μL of 20 ng/μL stock
T4 DNA ligase	0.5 μL

- Incubate at room temperature for 60 min.

Transformation:

- Transform the entire ligation mix into 100 μL of *E. coli* using the calcium chloride method. Make sure to pre-grow the cells in SOC or LB medium with no antibiotic prior

to plating. Plate on LB-agar containing the appropriate antibiotics. See the protocol page for "Transformation of *E. coli*."

Screening for correct clones:

- Pick 3-6 single, isolated colonies and inoculate each into a separate 14-mL tube containing 5 mL of liquid LB with the appropriate antibiotics. Remember: These are clones so take care not to cross-contaminate them from this point on! Grow the cultures overnight at 37 °C, shaking at 250-300 rpm. Make sure the tubes are tilted for maximal aeration.
- Purify plasmid from each clone, using a Qiagen Plasmid Miniprep Kit.
- Identify correct clones by PCR. See the PCR protocols page for "Insert verification with Vent." One primer must anneal to a region of the plasmid that is immediately outside the insert, and the second must anneal to a sequence within the insert. Analyze the PCR products on a 1% agarose gel.

Sequencing:

- Select 1 or 2 of the correct plasmid clones and measure the DNA concentration using the Nanodrop.
- Send 500-800 ng of each sample to Genewiz for sequencing. Ask Owen for the correct PO number to include in the paperwork.

Things to look up / think about:

Learn about the ligation mechanism as it is catalyzed by T4 ligase. How is this enzyme's mechanism different from that of *E. coli* ligase? What is the significance of the phosphate group at the DNA 5'-end?

During PCR screening, why must one primer match a sequence outside the insert and the second match a sequence within the insert?