

## Re-closing linearized plasmids

This protocol is used for generating mutant or truncated constructs.

### **Preparing the linearized plasmid:**

- See the PCR protocols page for “Whole plasmid amplification with Phusion.” Set-up the PCR mix with a final volume of 20  $\mu\text{L}$  (instead of 50). Make sure to adjust reagent volumes accordingly. **Note that the DpnI digestion step is critical.**
- After the DpnI digest, check 15  $\mu\text{L}$  of the sample on a 0.8% agarose gel.
- The rest of the sample should be immediately stored at  $-20\text{ }^{\circ}\text{C}$ .
- Note whether or not the primers are phosphorylated at the 5'-end.

### **For plasmids amplified using 5'-phosphorylated primers:**

- Set-up the following mix:

10x T4 DNA ligase buffer	1 $\mu\text{L}$
linearized DNA	1 $\mu\text{L}$
T4 DNA ligase	0.5 $\mu\text{L}$
ddH <sub>2</sub> O	7.5 $\mu\text{L}$

- Incubate at room temperature for 60 min.

### **For plasmids amplified using non-phosphorylated primers:**

- Treat the DNA with T4 polynucleotide kinase (PNK) to phosphorylate the 5'-ends:

10x T4 DNA ligase buffer	0.5 $\mu\text{L}$
linearized DNA	1 $\mu\text{L}$
T4 PNK	0.5 $\mu\text{L}$
ddH <sub>2</sub> O	3 $\mu\text{L}$

- Incubate at  $37\text{ }^{\circ}\text{C}$  for 30 min, then cool to room temperature.
- Add the following to the reaction mix:

ddH <sub>2</sub> O	4 $\mu\text{L}$
10x T4 DNA ligase buffer	0.5 $\mu\text{L}$
T4 DNA ligase	0.5 $\mu\text{L}$

- Incubate at room temperature for 60 min.

### **Transformation:**

- Transform the entire ligation mix into 100  $\mu\text{L}$  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. 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  $37\text{ }^{\circ}\text{C}$ , 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 or restriction digest. The appropriate screening method should distinguish between the desired plasmid and the parental plasmid. If using PCR, see the PCR protocols page for “Insert verification with Vent.” Analyze the PCR products or restriction digests 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?

What do you think is the smallest size of DNA (in kilobases) that can be ligated intramolecularly into a circular form?