

**Target Sequence Cloning Protocol**

*(Standard de-salted oligos are sufficient)*

**PX330-based plasmids, including PX458-462 – SpCas9 (or SpCas9n D10A nickase) + single guide RNA:**

To clone the guide sequence into the sgRNA scaffold, synthesize two oligos of the form:

 5’ – CACCGNNNNNNNNNNNNNNNNNNN – 3’

 3’ – CNNNNNNNNNNNNNNNNNNNCAAA – 5’

**PX260 and PX334 – SpCas9 (or SpCas9n D10A nickase) + CRISPR array + tracrRNA:**

To clone the guide sequence into the sgRNA scaffold, synthesize two oligos of the form:

 5’ – AAACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGT – 3’

 3’ – NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAAAAT – 5’

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**Oligo annealing and cloning into backbone vectors:**

1. Digest 1ug of plasmid with *Bbs*I for

 30 min at 37°C:

 1 ug Plasmid

 1 ul FastDigest *Bbs*I (Fermentas)

 1 ul FastAP (Fermentas)

 2 ul 10X FastDigest Buffer

 X ul ddH2O 20 ul total

2. Gel purify digested plasmid using

QIAquick Gel Extraction Kit and elute in EB.

3. Phosphorylate and anneal each pair of oligos:

 1 ul oligo 1 (100mM)

 1 ul oligo 2 (100mM)

 1 ul 10X T4 Ligation Buffer (NEB)

 6.5 ul ddH2O

 0.5 ul T4 PNK (NEB)

 10 ul total

Anneal in a thermocycler using the following parameters:

 37oC 30 min

 95oC5 min and then ramp down to 25oC at 5oC/min

4. Set up ligation reaction and incubate at room temperature for 10 min:

 X ul *Bbs*I digested plasmid

 from **step 2** (50ng)

 1 ul phosphorylated and annealed

 oligo duplex from **step 3** (1:200

 dilution)

 5 ul 2X Quickligation Buffer (NEB)

 X ul ddH2O

 10 ul subtotal

 1 ul Quick Ligase (NEB)

 11 ul total

5. (optional) Treat ligation reaction with PlasmidSafe exonuclease to prevent unwanted recombination products:

 11 ul ligation reaction from **step 4**

 1.5 ul 10X PlasmidSafe Buffer

 1.5 ul 10mM ATP

 1 ul ddH2O

 15 ul total

 Incubate reaction at 37C for 30 min.

6. Transformation