CRISPR | GENOME ENGINEERING TOOLBOX

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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’  - AAACNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGT      - 3’
3’  -      NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCAAAAT - 5’
```

* * * * *

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	ddH_2O
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 (100uM)
1 ul	oligo 2 (100uM)
1 ul	10X T4 Ligation Buffer (NEB)
6.5 ul	ddH_2O
0.5 ul	T4 PNK (NEB)
10 ul	total

Anneal in a thermocycler using the following parameters:

37°C	30 min
95°C	5 min and then ramp down to 25°C at 5°C/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	ddH_2O
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	exonuclease
15 ul	total

Incubate reaction at 37C for 30 min.

6. Transformation