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gRNA design and cloning with SapI into Loop plasmid L2_lacZgRNA-Cas9-CsA

Forked from gRNA design and cloning into Loop L2 plasmids (L2_gRNA-Cas9-CsA and L2_gRNA-CsA plasmids)

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1 Works for me dx.doi.org/10.17504/protocols.io.93wh8pe

OpenPlant Project

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ABSTRACT

This protocol explains how to design and clone the guide RNA target sequence into a L2 plasmid ready to accept the gRNA by cloning with SapI (L2 plasmid also contains a cassette to express Cas9)

Summary of design of gRNA and cloning into L2 with SapI

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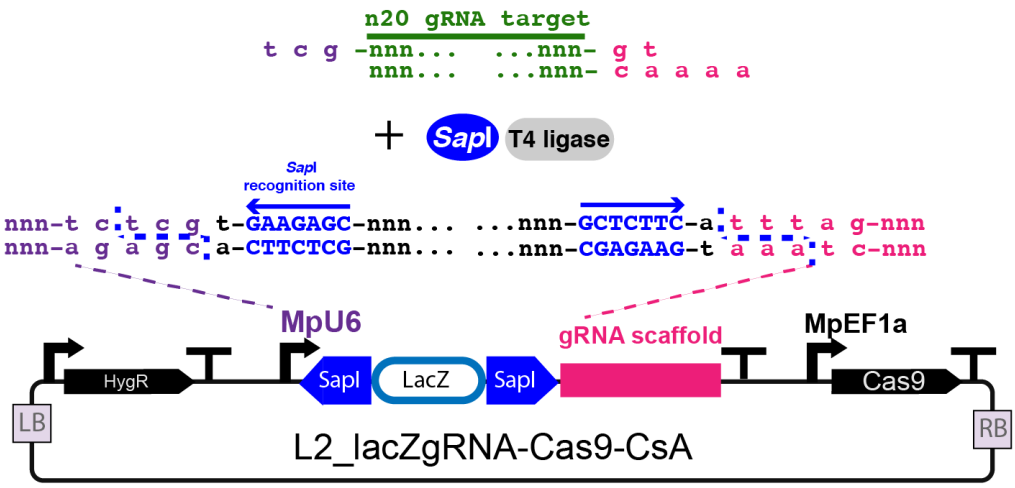

Design of oligos for gRNA SapI mediated cloning into L2_lacZgRNA-Cas9-CsA vector. The L2_lacZgRNA-Cas9-CsA SapI digested vector has AGC and TTT overhangs. Therefore, oligos for gRNA should be designed such that the forward strand has a 5' overhang of TCG and the reverse strand has a 5' overhang of gt-AAA (addition of “gt” nucleotides is necessary to reconstitute the full sequence of the gRNA scaffold in pink).Blue arrows: SapI recognition site. Blue dashed lines: SapI cleavage site. LacZ: lacZα cassette for blue-white screening of colonies.

2 gRNA oligo design

Order two oligos that contain the forward and reverse guide sequence plus the overhangs necessary for ligation (highlighted with bold) into L2_lacZgRNA-Cas9-CsA:

oligo F: 5'- **TCG**-NNNNNNNNNNNNNNNNNNN-**gt** 3'
oligo R: 5'-**AAAac**-NNNNNNNNNNNNNNNNNNN-3'

Note: Standard de-salted oligos are ok

3 Oligo annealing

Mix oligos with water as follow:

oligo F (100µM) 1µl
oligo R (100µM) 1µl
water 8µl
Total volume 10µl

Anneal in a thermocycler using the following parameters: 37^{o}C for 30 min, 95^{o}C for 5 min and then ramp down to 25^{o}C at 5^{o}C per min. After annealing the gRNA can be directly cloned into L2_lacZgRNACas9-CsA plasmid without the need of any further processing (step 4).

4 **Cloning into backbone vector**

In a 0.2 mL tube set up the following reaction:

Component	Volume (µL)
Sterile water	5
10x Tango buffer (Thermo Fisher)	1
1 mg/mL bovine serum albumin (NEB)	0.5
T4 DNA ligase (5 U/µL) (Thermo Fisher)	0.25
10mM ATP (SIGMA)	1
SapI (LguI) (5 U/µL) (Thermo Fisher)	0.25
L2_lacZgRNACas9-Csa (25-50 ng)	1
annealed oligo	1
Final volume	10

- Place samples on the thermocycler and incubated using the following program:

Assembly: 15 cycles: 3 minutes at 37^{o}C and 4 minutes at 16^{o}C
Termination: 5 minutes at 50^{o}C and 10 minutes at 80^{o}C

- Transform chemically competent using 1 µL of reaction and plate on LB agar plates with 100 µg/mL spec and X-gal 40. Incubate at 37 oC for 16 h.

- Confirm with sequencing