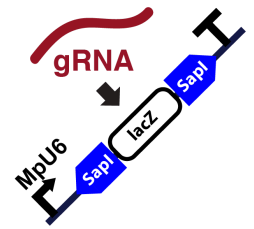


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## 🌐 gRNA design and cloning into Loop L2 plasmids (L2\_gRNA-Cas9-CsA and L2\_gRNA-CsA plasmids)

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**Protocol status:** Working

**We use this protocol and it's working**

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## Abstract

This protocol explains how to design clone the guide RNA (gRNA) sequence into the L2 plasmids ready to accept the gRNA sequence by cloning with SapI.

We have two versions of the plasmid, with or without Cas9: the L2\_gRNA-Cas9-CsA and L2\_gRNA-CsA plasmids.

With L2\_gRNA-Cas9-CsA you transform wild-type sporelings.

With L2\_gRNA-CsA you transform sporelings of a line expressing Cas9.

## 1 **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\_gRNA-CAs9-CsA or L2\_gRNA-CsA plasmids:

**oligo F:** 5'- **TCG**-NNNNNNNNNNNNNNNNNNNNNNNN-**gt** 3'

**oligo R:** 5'-**AAAac**-NNNNNNNNNNNNNNNNNNNNNNNN-3'

Note: Standard de-salted oligos are ok

## 2 **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°C for 30 min, 95°C for 5 min and then ramp down to 25°C at 5°C per min. After annealing the gRNA can be directly cloned into L2\_gRNA-CAs9-CsA or L2\_gRNA-CsA plasmids without the need of any further processing.

## 3 **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/ $\mu$ L) (Thermo Fisher)	0.25
10mM ATP (SIGMA)	1
SapI (LguI) (5 U/ $\mu$ L) (Thermo Fisher)	0.25
L2_393-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°C and 4 minutes at 16°C

Termination: 5 minutes at 50°C and 10 minutes at 80°C

- Transform chemically competent using 1  $\mu$ L of reaction and plate on LB agar plates with 100  $\mu$ g/mL spec and X-gal 40. Incubate at 37 oC for 16 h.
- Confirm with sequencing