

Eftychis Frangedakis¹, marta tomaselli², Susana Sauret-Gueto³

1 Works for me [dx.doi.org/10.17504/protocols.io.5jqg4mw](https://doi.org/10.17504/protocols.io.5jqg4mw)

OpenPlant Project



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_qRNA-Cas9-CsA you transform wild-type sporelings.

With L2_gRNA-CsA you transform sporelings of a line expressing Cas9.

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-NNNNNNNNNNNNNNNNNNNNNN-qt 3'

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

Note: Standard de-salted oligos are ok

Mix oligos with water as follow:

oligo F (100μM) 1μl

oligo R (100μM) 1 μl

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/μL) (Thermo Fisher)	0.25
10mM ATP (SIGMA)	1
SapI (LgI) (5 U/μL) (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 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



This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited