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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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We use this protocol and it's working

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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

The diagram illustrates the components and assembly of the L2_lacZgRNA-Cas9-CsA construct. At the top, a 20 bp gRNA target sequence is shown: **n20 gRNA target**. The sequence is: **t c g -nnn... ...nnn- g t** (top line) and **nnn... ...nnn- c a a a a** (bottom line). This is combined with **SapI** (represented by a blue oval) and **T4 ligase** (represented by a grey oval) to form the final construct.

The final construct, **L2_lacZgRNA-Cas9-CsA**, is shown as a linear map. It starts with a **LB** (Left Border) element, followed by a **HygR** (hygromycin resistance) gene. A **T** (terminator) element follows. Then, the **MpU6** promoter drives the expression of the **SapI** recognition site (blue hexagon), followed by the **LacZ** gene (blue oval). Another **SapI** recognition site (blue hexagon) is present. This is followed by the **gRNA scaffold** (pink rectangle). A **T** (terminator) element follows. The **MpEF1a** promoter drives the expression of the **Cas9** gene (black rectangle). The construct ends with a **RB** (Right Border) element.

Detailed sequence alignment for the gRNA scaffold assembly:

Top strand (5' to 3'): nnn-t c t c g t-**GAAGAGC**-nnn... ...nnn-GCTCTTC-a-t t t a g-nnn

Bottom strand (3' to 5'): nnn-a g a g c-a-**CTTCTCG**-nnn... ...nnn-CGAGAAG-t a a a t c-nnn

The **SapI** recognition site is highlighted in blue: **GAAGAGC** (top) and **CTTCTCG** (bottom). The **gRNA scaffold** is highlighted in pink: **GCTCTTC** (top) and **CGAGAAG** (bottom).

Protocol for design of gRNA and cloning into L2 with SapI

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 R: 5'-**AAAac**-NNNNNNNNNNNNNNNNNNNN-3'

3 Oligo annealing

oligo F (100μM) 1μl
oligo R (100μM) 1μl
water 8μl

Total volume 10 μ L

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_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 (μ L)
Sterile water	5
10x Tango buffer (Thermo Fisher)	1
1 mg/mL bovine serum albumin (NEB)	0.5
T4 DNA ligase (5 U/ μ L) (Thermo Fisher)	0.25
10mM ATP (SIGMA)	1
SapI (LgI) (5 U/ μ L) (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°C and 4 minutes at 16°C

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

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

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- Confirm with sequencing