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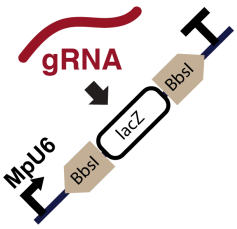
gRNA design and cloning with BbsI into Loop plasmid L1_lacZgRNA-Ck2/3 V.1



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Eftychis Frangedakis[1], Marta Marta Tomaselli[2], Susana Sauret-Gueto[2]
[1]University of Cambridge; [2]Plant Sciences, University of Cambridge, OpenPlant

OpenPlant Project

Susana Sauret-Gueto
Plant Sciences, University of Cambridge, OpenPlant

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Protocol status: Working
We use this protocol and it's working

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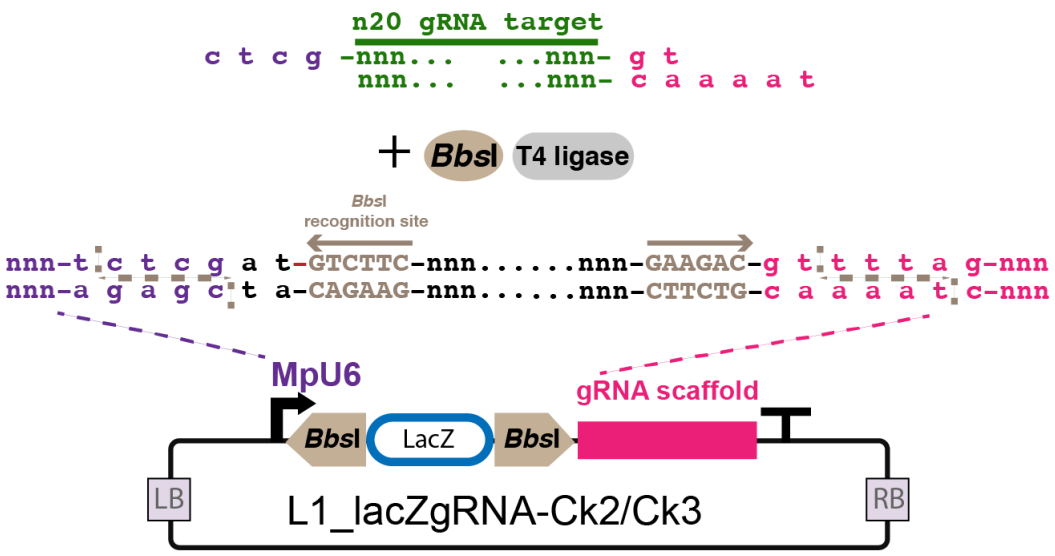
Abstract

This protocol explains how to design and clone the guide RNA target sequence into a L1 plasmid ready to accept the gRNA by cloning with BbsI. L1 plasmids are L1_lacZgRNA-Ck2 and L1_lacZgRNA-Ck3.

If one gRNA target sequence is cloned into the Ck2 plasmid and another one into Ck3 one, the two L1_gRNA transcription units can be combined with an antibiotic resistance transcription unit and a MpEF1α:Cas9 transcription unit via **L2 SapI Loop assembly.** This allows for dual gRNA editing.

Summary of design of gRNA and cloning into L1 with BbsI

1



Design of oligos for gRNA BbsI mediated cloning into L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3 vectors. The L1_lacZgRNA-Ck2/3 BbsI digested vectors have GAGC and TTTA overhangs. Therefore, oligos for gRNA should be designed such that the forward strand has a 5' overhang of CTCG and the reverse strand has a 5' overhang of gt-AAAT (addition of “gt” nucleotides is necessary to reconstitute the full sequence of the gRNA scaffold in pink). Light brown arrows: BbsI recognition site. Light brown dashed lines: BbsI cleavage site. LacZ: lacZα cassette for blue-white screening of colonies.

Protocol for design of gRNA and cloning into L1 with BbsI

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 L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3:

oligo F: 5′- **CTCG**-NNNNNNNNNNNNNNNNNNN-**gt** 3′
oligo R: 5′- **TAAAac**-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 L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3 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	6
	10x T4 ligase buffer (NEB)	1
	1 mg/mL bovine serum albumin (NEB)	0.5
	T4 DNA ligase (5 U/µL) (Thermo Fisher)	0.25
	SapI (LguI) (5 U/µL) (Thermo Fisher)	0.25
	L1_lacZgRNA-Ck2 or L1_lacZgRNA-Ck3 (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 µg/mL for blue-white screening. Incubate at 37^{o}C for 16 h.

- Confirm with sequencing