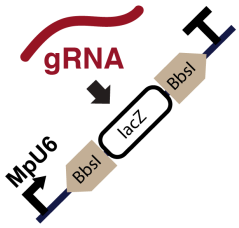
Dec 04, 2019 Version 1

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



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

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Keywords: l1-grna transcription unit, cas9 transcription unit via l2 sapi loop assembly, grna target sequence, guide rna target sequence, plasmid l1-laczgrna, cas9 transcription unit, antibiotic resistance transcription unit, dual grna, grna design, grna, rna, cloning, l2 sapi loop assembly, bbsi into loop, mpef1α, cas9

Abstract

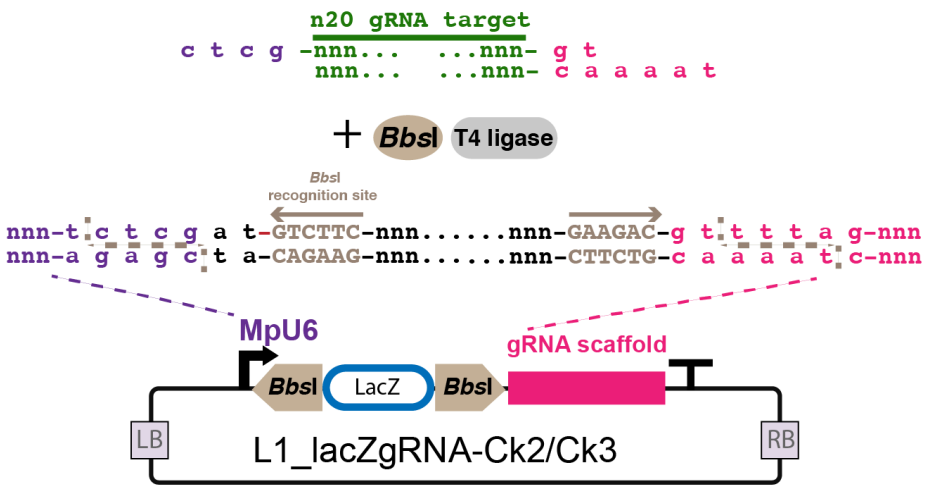
This protocol explains how to design and clone the guide RNA target sequence into a L1 plasmid ready to accept the gRNA by cloning with BbsI. L1 plasmids are L1_lacZgRNA-Ck2 and L1_lacZgRNA-Ck3.

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.

Troubleshooting

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