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

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CRISPR gRNAs cloning

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ABSTRACT

This protocol details the procedure of CRISPR gRNAs cloning.

ATTACHMENTS

gfccbg5jf.pdf



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gRNA oligonucleotides design

1 To design your gRNAs, use CRISPick portal

(https://portals.broadinstitute.org/gppx/crispick/public).

1.1 Order the Oligos with specific overhangs for BsmBI cloning.

1.2 Insert the designed 20bp target gRNA sequence between the overhangs.

Forward oligo: 5' CACCG......20 bp target......-3'

Reverse oligo: 5' AAAC.....20 bp........C 3'

gRNA oligonucleotides cloning

- 2 Preparation of the gRNA oligonucleotides.
 - **2.1** Spin oligonucleotide tubes briefly.



- 2.2 Dilute to [M] 100 micromolar (µM) solution with water.
- **2.3** Vortex, leave for some minutes, and vortex again.



2.4 Annealing of oligonucleotides:



- [M] 100 micromolar (μM) of oligo A (forward)
- [M] 100 micromolar (μM) of oligo B (reverse)
- 4 2 µL 10x NEB buffer 2
- water up to 🚨 20 µL total reaction volume

2.5 Denature at \$\mathbb{g} 95 \circ for \oldots 00:05:00 , then cool down slowly.

5m

Note

Recommended: Turn the heating block of and leave the tubes in it for 02:00:00 - 03:00:00

2.6 When they have reached Room temperature, spin down.



3 Prepare the assembly reaction for each oligonucleotide in individual PCR tubes containing:



- 1. 4 100 ng backbone of lentiviral plasmid of choice (make sure it includes the AmpR gene for selection)
- 2. \triangle 1 μ L of annealed gRNA oligonucleotide from step 1
- 3. Δ 1 μL BsmBI/Esp3I restriction enzyme. FAST DIGEST
- 4. 🚨 1 μL T4 DNA ligase
- 5. Δ 2 μL 10x T4 ligase buffer (to a final concentration of 1x)
- 6. Nuclease-free water up to 4 20 µL total reaction volume
- 3.1 Incubate the reaction in a thermal cycler with the following conditions:

11



- 10 cycles 👏 00:05:00 at 🖁 37 °C .
 - ♦ 00:10:00 at \$\mathbb{g}\$ 22 °C .
- Hold for 🕙 00:30:00 at 🖁 37 °C .
- Hold for 👏 00:15:00 at 🖁 75 °C .
- Keep at 🔓 4 °C .

4

Plasmid transformation & preparation Thaw Stbl3 or homemade top10 competent bacteria On ice.



4.2 Mix a little by tapping the tube carefully a couple of times.



4.3 Keep **§** On ice for **(S)** 00:30:00 .

30m

4.4 To transform, dip the tubes in a 42 °C water bath for exactly 00:00:45.

45s

4.5 Put the tubes back On ice for 00:02:00.

2m

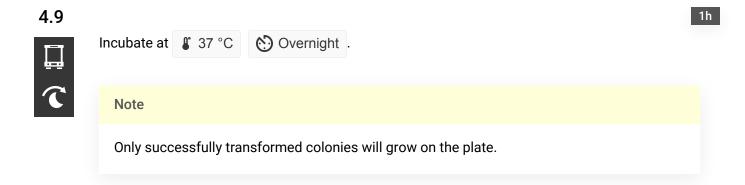
- 4.6 Transfer the bacteria to Δ 250 μL pre-warmed or Room temperature soc media in a ventilated 15 ml falcon tube.
- 4.7 Incubate at \$\mathbb{8}^* 37 \circ C with shake for \infty 01:00:00 \tag{5}.

1h

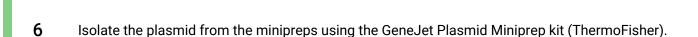
5



4.8 Spread everything on pre-warmed ampicillin+ agar plates.



The day after, pick up 3 different colonies for each of the plasmids from the agar plates and prepare 3 minipreps. Incubate the minipreps Overnight on shake at 37 °C.



- **6.1** Measure the DNA concentration.
- **6.2** Digest the DNA using the AfIII (BspTI) restriction enzyme to linearize the plasmid.

Oct 15 2024

1h

Note

This restriction site is part of the LTR found in lentiviral plasmids.

- **6.3** Check for the correct plasmid size on 1% agarose gel.
- **6.4** If the plasmids have the correct size, send for sequencing 1 or 2 for each cloned gRNA..
- If the sequencing confirms the correct plasmid sequence, use one of the sequenced miniprep for each gRNA to prepare a maxiprep. Incubate the maxipreps Overnight at \$37 °C.



- 8 Isolate the plasmid from the maxipreps using the NucleoBond Xtra Midi Plus Ef (ThermoFisher).
 - **8.1** Measure the DNA concentration.
 - 8.2 Digest the DNA using the AfIII (BspTI) restriction enzyme to linearize the plasmid.



8.3 Check for correct plasmid size on 1% agarose gel, and send for sequencing.

