CRISPR gRNAs cloning

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Anita Adami: CRISPR gRNAs cloning. **protocols.io**
https://protocols.io/view/crispr-grnas-cloning-b32qqqdw

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gRNA oligonucleotides design 30m

1 30m

To design your gRNAs, use CRISPick portal (https://portals.broadinstitute.org/gppx/crispick/public).

Order the Oligos with **specific overhangs** for BsmBI cloning. 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 6h 30m

2 3h 30m

Preparation of the gRNA oligonucleotides

Spin oligonucleotide tubes briefly.
Dilute to 100µM solution with water.
Vortex, leave for some minutes, and vortex again.

Annealing of oligonucleotides:

- 100 µM of oligo A (forward)
- 100 µM of oligo B (reverse)
- 2 µl 10xNEB buffer 2
- water up to 20 µl total reaction volume

Denature at 95° for 5 min, then cool down slowly.
Recommended: turn the heating block of and leave the tubes in it for 2-3 h.
When they have reached RT, spin down.

3 3h

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

1. 100 ng backbone of lentiviral plasmid of choice (make sure it includes the AmpR gene for selection)
2. 1 µl of annealed gRNA oligonucleotide from step 1
3. 1 µl BsmBI/Esp3I restriction enzyme. FAST DIGEST
4. 1 µl T4 DNA ligase
5. 2 µl 10x T4 ligase buffer (to a final concentration of 1x)
6. Nuclease-free water up to 20 µl total reaction volume

Incubate the reaction in a thermal cycler with the following conditions:

10 cycles	5 min at 37 °C
	10 min at 22 °C
Hold for	30 min at 37 °C
Hold for	15 min at 75 °C
Keep at 4 °C	

plasmid transformation & preparation 5d

4 Thaw Stbl3 or homemade top10 competent bacteria on ice. 1d

Add 2 µl of the ligation reaction from step 2 to the bacteria on ice. Mix a little by tapping the tube carefully a couple of times.
Keep on ice for 30 min.

To transform, dip the tubes in a 42°C water bath for exactly 45 s, then put the tubes back in ice for 2 min.

Transfer the bacteria to 250 µl pre-warmed or room temperature soc media in a ventilated 15 ml falcon tube and incubate at 37 °C with shake for 1 h.
Spread everything on pre-warmed ampicillin+ agar plates and incubate at 37 °C overnight.
Only successfully transformed colonies will grow on the plate.

5 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. 1d

6 Isolate the plasmid from the minipreps using the GeneJet Plasmid Miniprep kit (ThermoFisher), measure the DNA concentration, and digest the DNA using the AflII (BspTI) restriction enzyme to linearize the plasmid (this restriction site is part of the LTR found in lentiviral plasmids). Then, check for the correct plasmid size on 1% agarose gel. 1d

If the plasmids have the correct size, send for sequencing 1 or 2 for each cloned gRNA.

7 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. 1d

8 Isolate the plasmid from the maxipreps using the NucleoBond Xtra Midi Plus Ef (ThermoFisher), measure the DNA concentration, and digest the DNA using the AflII (BspTI) 1d

restriction enzyme to linearize the plasmid. Check for correct plasmid size on 1% agarose gel, and send for sequencing.