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Cloning guides to lentiCRISPR v2 V.1

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

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Abstract

I have been struggling with cloning guide RNA sequences to the lentiCRISPR vector (**https://www.addgene.org/52961/**) for months now and have finally figured out a method that consistently works for me, so I felt like I should share this information with whomever is struggling as much as I did!

Troubleshooting

Before start

Make Stbl3 chemically competent cells.

Vector preparation:

1 Digest the lentiCRISPRv2 vector:
3µg vector
2µl 10X Tango buffer (Thermo Fisher)
1µl 20mM DTT
1-1.5µl Esp3I (Thermo Fisher)
Water to 20µl

In a thermocycler:
37°C for 4 hours, inactivate at 65°C for 20 mins, keep at 4°C.
SKIP the alkaline phosphatase step.

2 Use a gel purification/PCR cleanup kit (Qiagen) **WITHOUT** running on a gel.
Optional: run ~200ng of the purified vector on a gel to verify digestion.

Guide insert preparation:

3 Anneal and phosphorylate gRNA oligos pair:
1µl of each oligo (100µM stock)
1µl 10X T4 ligation buffer (not PNK buffer)
0.5µl T4 PNK
Water to 10µl

In a thermocycler: 37ºC for 30 mins, 95ºC for 5 mins, ramp down to 25ºC at 0.1ºC/sec (or 5-6ºC/min).
Optional: keep at 4ºC.

4 Serially dilute the annealed oligos to 1:500

Ligation:

5 Out of ligation at a vector:insert molar ratios of 1:5, 1:10, 1:20, I found that 1:5 works best.

50ng vector
1.5µl 10X T4 ligation buffer (NEB)
1µl T4 ligase (NEB)
2µl diluted oligos (1:500)
Water to 15µl

Incubate at RT for 1-2 hours.

Transformation:

6 Transform 5µl of the ligation reaction to 50µl Stbl3 chemically competent cells.

If you incubate the Stbl3 cells at 30°C the colonies will be VERY small so look for them carefully. Incubating them at 37°C didn't result in LTR recombination in my hands.

Colony PCR:

7 Replica plate colonies on a new LB-Amp plate prior to inserting the tip to 10µl water.
Mix well/ vortex
Boil at 98°C for 10 mins

8 Using primers upstream and downstream of the guide insert sequence, perform colony PCR:
F primer: gca tat acg ata caa ggc tgt tag aga ga
R primer: gag cca gta cac gac atc act t

		µl
	10X buffer	2
	10mM dNTPs	0.4

	F primer(10uM)	0.3
	R primer(10uM)	0.3
	Boiled colony	0.5
	Taqpol	0.1
	ddH2O	16.4
	Total:	20

* Can be scaled up or down.

PCR program:

	1	94°C	5 min

	2	94°C	30 sec
	3	54°C	1 min
	4	72°C	1 min/Kb
	5	Go to step 2	X30
	6	72°C	5 min
	7	4°C	Hold

9 Run on a gel -
product of positive colonies: 500 bp
product of negative colonies: 2500 bp

10 Send ~2 colonies per guide for sequencing with U6_fwd commercial primer.