Cloning guides to lentiCRISPR v2

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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!

Citation: Amit Weiner Cloning guides to lentiCRISPR v2. **protocols.io** dx.doi.org/10.17504/protocols.io.qx3dxqn
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Before start

Make Stbl3 chemically competent cells.

Protocol

Vector preparation:

Step 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.

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:

Step 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.

Step 4.
Serially dilute the annealed oligos to 1:500

Ligation:

Step 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:

Step 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:

Step 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

Colony PCR:

Step 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
Taq pol	0.1
ddH_2O	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

Colony PCR:

Step 9.

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

Colony PCR:

Step 10.

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