Protocol: Arrayed cloning of dual sgRNAs into lentiviral vectors

This protocol works for low or medium throughput cloning of programmed pairs of sgRNAs into a lentiviral expression vector. The guide expression cassette of mU6-CR3-hU6-CR1 uses different U6 and sgRNA constant regions in the two positions in order to prevent intramolecular lentiviral recombination.

The strategy uses four components:

1) sgRNA expressing lentiviral vectors (note: it is not necessary to use a BsmBI- vector for this protocol. This feature is only important for pooled dual-guide library cloning.)

plasmid	U6 promoter	constant region	capture sequence	Pol-II promoter	Selectable marker
pJR85	mU6	modified CR1	CS1 in stem loop	EF1alpha (no UCOE)	PURO-BFP (BsmBI-)
pJR100	mU6	modified CR1	CS1 in stem loop	UCOE-EF1alpha	PURO-BFP (BsmBI-)
pJR101	mU6	modified CR1	CS1 in stem loop	UCOE-EF1alpha	PURO-GFP (BsmBI-)
pJR103	mU6	CR1	none	UCOE-EF1alpha	PURO-BFP (BsmBI-)
pJR104	mU6	CR1	none	UCOE-EF1alpha	PURO-GFP (BsmBI-)

2) top and bottom oligos annealed for position A sgRNA targeting sequence (see excel template)

3) CR3-hU6 insert

plasmid	U6 promoter	constant region	capture sequence
pJR89	hU6	modified CR3	CS1 in stem loop
pJR97	hU6	CR2	none
pJR98	hU6	CR3	none

4) top and bottom oligos annealed for position B sgRNA targeting sequence (see excel template)

Capture sequences are used for 3' direct capture Perturb-seq. For all other experiments, it is not necessary to use capture sequences in the sgRNA constant region. If you are cloning many sgRNAs, I recommend buying your oligos in pre-suspended at 100uM in a plate in an arrayed format that is easy to combine top and bottom oligo by multichannel. Currently, only ~30-50% of colonies contain the desired sequence (although I am working on troubleshooting this by either adjusting the ratio of vector to insert or using phosphatase treated vector).

Step 1: Annealing of sgRNA targeting sequences:

48µL IDT duplex buffer
1µL 100uM top oligo
1µL 100uM bottom oligo
50µL total reaction volume

Anneal top and bottom oligo for position A. Anneal top and bottom oligo for position B. Incubate at 95°C for 5min in a PCR machine. Let oligos gradually anneal while cooling to RT (take the plate out and set it on your bench or use a cooling ramp on a PCR machine to cool the plate).
Make a 1:20 dilution of annealed oligos in ddH_2O.
Annealed oligos can be stored a -20°C and are stable through at least 2-3 freeze thaws.

Step 2: Insert digest

5ug pJR98
10µL 10x 3.1 buffer
5µL NEB BsmBI
xx µL ddH_2O
100µL total reaction volume

Incubate at **55 C** for 1+ hour. Gel extract the desired 400 bp insert.

Step 3: Vector digest

5ug pJR103
10µL 10x 2.1 buffer
4µL NEB BlpI
4µL NEB BstXI
xx µL ddH_2O
100µL total reaction volume

Incubate at **37 C** for 1+ hour. Gel extract the desired 9kb vector.

Ligation:

100ng digested vector backbone (digested with BstXI and BlpI)
20ng digested insert (digested with BsmBI)
6µL 1:20 diluted annealed oligo pair A
6µL 1:20 diluted annealed oligo pair B
2µL fresh 10X T4 ligase buffer
1µL T4 ligase
xx µL ddH_2O
20µL total reaction volume

Incubate at RT for 1-4hrs or 16C overnight.

Transform to DH5α bacteria:

Pre-warm LB agar + amp or carb plate(s) at 37°C.
Combine 20µL cells + 1µL ligation; incubate 30min on ice.
Heat shock cells at 42°C for 45 sec (this can be done in tubes with a water bath or a PCR machine with 96 well plates- prewarm the PCR block to 42C and then set the plate in).
Put immediately back on ice for ~1 min.

For low throughput cloning

add 100µL LB to transformation and recover at 37°C shaking for 1hr.
Plate 50µL of the transformation
Incubate at 37°C overnight.

For higher throughput cloning

Immediately plate all 20uL of transformed bacteria onto a spot on a partitioned plate and streak off the spot to single cell dilution. I use as little as 4uL of bacteria to save money.

Pick 4 colonies per sgRNA pair and miniprep (use Zymo Research Zyppy-96 (D4041) for 96 well plates minipreps format). Or use standard mini preps to prep plasmid DNA. As stated above, only ~30-50% of colonies contain the desired sequence although I am working on troubleshooting this by either adjusting the ratio of vector to insert or using phosphatase treated vector.

Sanger sequence with oBA420 (5'- cagcacaaaaggaaactcacc -3')