Protocol for making double-sgRNA constructs using pCFD4.
Modified from http://www.crisprflydesign.org/grna-expression-vectors/
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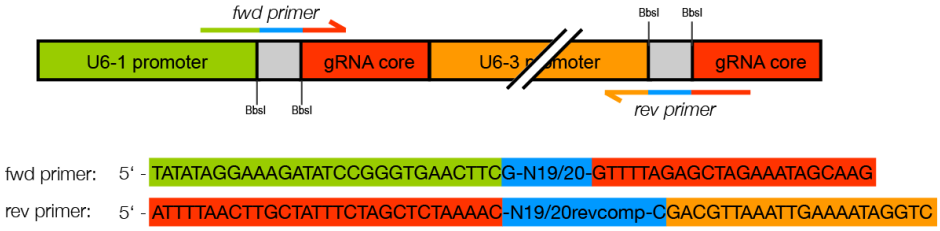
Cloning tandem gRNA expression vectors with pCFD4



We use ligation independent cloning to introduce two protospacer sequences into pCFD4. We often use Gibson Assembly (available as kit from NEB), but SLIC (Li and Elledge, Nat Methods, 2007) works equally well.

Step 1: Order the fwd and rev primers shown in the figure above. If your protospacer sequence starts with a G then N will be 19, otherwise N will be 20.

Step 2: Run a PCR using a high-fidelity polymerase and pCFD4 as a template.

Step 2 details:

Set up a PCR using Phusion HS Taq according to this ratio:

Reagant	volume
5X Phusion HF Buffer	10
2 mM dNTPs mix	5
10 uM F primer	2.5
10 uM R primer	2.5
pCFD4 plasmid (1:100 of a miniprep, ~5-10ng)	2
Phusion HS Taq	0.5
H20	27.5
Total	50

Run the following PCR program:

1. 95C – 30s
2. 95C – 10s
3. 61C – 30s

4. 72C – 1min
5. Go to step 2 for 35 more times.
6. 72C – 10 min
7. 4C – forever.

Step 3:
Digest pCFD4 plasmid with BbsI enzyme:

Component	µL
pCFD4 plasmid	2 µg
10x NEB4 Buffer	4
Bbs1	2
dH20	to 40 µL

Step 4:
Run the PCR reaction from Step 2 and the digested BbsI from Step 3 a 1% agarose gel, and gel-purify the bands using the QIAgen purification kit, eluting into 30µL of elution buffer.

The PCR products are ~600bp, and the digested plasmid is ~6.4kb.

Step 5:
Combine the PCR product and the pCFD4 backbone using Gibson. Combine the following in a PCR reaction tube:

Component	µL
digested pCFD4	4
PCR product	1
Gibson Mix	5

Place tube at 50C for 1 hour.

Step 6:
Transform 2 µL of the Gibson reaction into 25µL of TOP10 chemically-competent cells using a 30-second heat shock at 42C. Add 250 of LB, grow at 37C for 1 hour, then plate the entire reaction on LB-Carb (or LB-Amp) plate.

Step 7:
Miniprep 2 colonies per reaction and sequencing using the following primer to make sure the reaction worked: GACACAGCGCGTACGTCCTTCG.

Step 8:
Prepare these plasmids for pooled injections (4-5 plasmids per injection) into attP40 site in a vermillion- background.