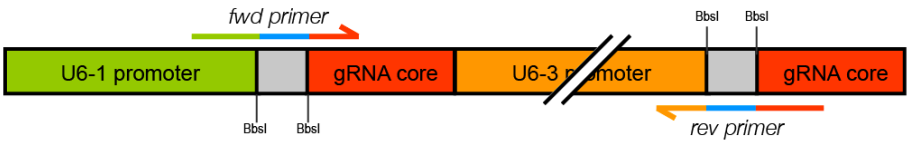
Cloning tandem gRNA expression vectors with pCFD4



fwd primer: 5' - TATATAGGAAAGATATCCGGGTGAACTTCG-N19/20-GTTTTAGAGCTAGAAATAGCAAG

rev primer: 5' - ATTTTAACTTGCTATTTCTAGCTCTAAAAC-N19/20revcomp-CGACGTTAAATTGAAAATAGGTC

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.

***Important**: Several users have found this PCR to be susceptible to failure when the wrong annealing temperature is used. We recommend using an annealing temperature of 61°C in combination with the Q5 PCR master mix from NEB. Optimal Ta might vary depending on the target sequence incorporated into the primer and the PCR kit used.*

Step 3: Digest pCFD4 with BbsI.

Step 4: Run the PCR reaction and the digested vector on a 1% agarose gel and cut out the DNA bands (PCR 600bp; Backbone 6.4kb).

Step 5: Gel purify insert and backbone.

Step 6: Assemble the plasmid by either Gibson Assembly (NEB, follow manual) or SLIC (Li and Elledge, Nat Methods, 2007).

Step 7: Transform into competent bacteria. Plate on Ampicillin plates.

Step 8: Verify insert by sequencing. We use the following sequencing primer: **GACACAGCGCGTACGTCCTTCG**; which allows sequencing of both gRNAs in one reaction. We typically test 2-4 colonies per cloning.