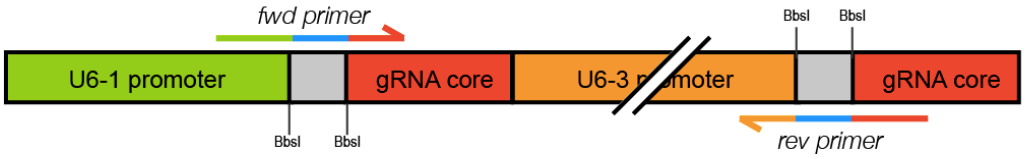
Cloning a tandem gRNA insect expression vector (pCFD4)

This protocol is for cloning two sgRNAs into an insect expression vector, pCFD4, for injection into *Drosophila* embryos for the generation of a CRISPR mutant. It is adapted from a protocol from crisprflydesign.org and uses phusion polymerase, the restriction enzyme BbsI, Thermo Scientific Gel Extraction kit (K0691) and the Gibson Assembly kit (E5510S), NEB 10-beta competent cells, and GeneJET Plasmid Miniprep Kit.

Design Oligos

1. Using the template below to design forward and reverse primers. Each primer contains a different target site. The blue sequence should be 20 nt in total and may need the first bp to be changed to G (fwd) or C (rev).



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

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

PCR gRNA Insert

2. Prepare working stocks of primers with water to a concentration of 2 µM.
3. Set up the following 30 µl PCR reaction using pCFD4 as a template:

5x High Fidelity Phusion Buffer	6 µl
DMSO	1 µl
dNTPs	0.6 µl
Fwd primer (2 µM)	1.5 µl
Rev primer (2 µM)	1.5 µl
300 ng template (pCFD4)	variable
Phusion polymerase	0.3 µl
Water	to 30 µl

4. Anneal and extend using the thermocycler:

1: 98°C	2 min
2: 98°C	10 sec
3: 55-63°C	20 sec (increase by 0.5°C/cycle)
4: 72°C	15 sec
repeat 2-4 for 16 cycles	
5: 98°C	10 sec
6: 72°C	25 sec

repeat 5-6 for 15 cycles

7: 72°C	5 min
8: 12°C	hold

Digest pCFD4

5. While PCR runs, set up the following 10 µl digestion reaction of pCFD4 (GL plasmid stock #122):

300 ng template (pCFD4 vector)	variable
NEB 2.1 buffer	1 µl
BbsI (from NEB)	1 µl
Water	to 10 µl

Note: There is both a BbsI (R0539S) and BbsI-HF (R3539S). BbsI must be stored at -80°C and used with 2.1 buffer, while BbsI-HF is stored at -20°C and used with cutsmart buffer.

6. Incubate at 37°C for at least 1 hr.

Gel Extract and Purify (Thermo Scientific Gel Extraction Kit)

7. Run both the PCR product from steps 3-4 and the digested vector from steps 5-6 on a 1% TAE agarose gel. The PCR product should be ~600 bp and the digested vector should be ~7000 bp.
8. Cut out bands and place into 1.5 ml tubes.
9. Add 1:1 volume pre-warmed binding buffer (e.g. add 100 µl of binding buffer for every 100 mg of agarose gel).
10. Incubate at 55°C for 10 min with occasional vortexing until gel has fully dissolved.
11. Transfer gel solution to GeneJET purification column. Centrifuge in desktop centrifuge for 30 seconds. Discard flow through.
12. Add 700 µl wash buffer to the column. Centrifuge for 30 seconds. Discard flow through.
13. Centrifuge empty column for an additional minute to dry.
14. Add 15 µl elution buffer to the column. Incubate for 2-3 minutes.
15. Centrifuge for 30 seconds to elute DNA.
16. Nanodrop 1 µl of sample to determine concentration.

Plasmid Assembly with Gibson Assembly Kit

17. Use NEBBioCalculator (https://nebiocalculator.neb.com/#!/ligation) online to determine the ratio of vector DNA mass: insert DNA mass. A good amount of vector mass is ~75 ng and a good ratio is 2:1 or 3:1. Insert DNA length is ~600 bp and vector DNA length is ~7000 bp.

18. Set up the following reaction on ice in a PCR strip:

Insert DNA	~75 ng
Template DNA	~20 ng
Gibson Master Mix	10 µl
Water	to 20 µl

For a positive control:

NEB Builder Positive Control	10 µl
Gibson Master Mix	10 µl

19. Incubate at 50°C for 1 hr. Place on ice for immediate transformation or store at -20°C.

Transformation

20. Thaw NEB 10-beta competent cells on ice for 5 minutes.
21. Transfer 50 µl of cells to a 1.5 ml tube.
22. Add 2 µl of the Gibson reaction to the cells. Gently mix by pipetting.
23. Incubate on ice for 30 minutes.
24. Heatshock at 42°C for 30 seconds.
25. Incubate on ice for 5 minutes.
26. Add 950 µl room temperature SOC outgrowth medium.
27. Shake at 37°C for 1 hr. Warm ampicillin plates at 37°C.
28. Plate cells on amp plates. Reaction is efficient. 50-250 µl is recommended.
29. Incubate plates overnight at 37°C.

Mini-prep colonies

30. Choose several colonies and prep 3 ml cultures. Allow to grow at 37°C overnight.
31. Spin down cells at 8000 rpm for 2 min and discard LB broth.
32. Add to the pelleted cells 250 µl Resuspension solution and vortex.
33. Add 250 µl Lysis solution and invert the tube 4-6 times.
34. Add 350 µl Neutralization solution and invert the tube 4-6 times.
35. Centrifuge for 5 min at max speed.
36. Transfer the supernatant to the Termo Scientific GeneJET spin column. Centrifuge 1 min. Discard flow through.
37. Add 500 µl Wash solution and centrifuge for 30 seconds. Discard flow through.
38. Repeat wash step.
39. Centrifuge empty column for 1 min.
40. Transfer the column to a new 1.5 ml tube.

Add 50 µl nuclease-free water directly to the column and incubate for 2-3 minutes.

41. Centrifuge 2 minutes and collect the flow through.
42. Nanodrop 1 µl to determine concentration.

Sequencing

43. Set up a sequencing reaction using the Grimes Lab primer GL296 (pCFD4 sequencing primer). This will allow verification that both guides were inserted properly.
44. For Genewiz sanger sequencing:

template	~800 ng
water	to 10 µl
primer	5 µl