Adapted from "Cas9-Based Genome Editing in Drosophila"
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When designing sgRNA oligos, be sure to include the relevant 4-bp overhangs to allow ligation into the digested vector and when using a U6 expression plasmid, also include an additional G at the start of the sgRNA sequence to initiate transcription

Materials

- Complementary oligos carrying sgRNA target sequence (not including PAM)
- Suitable plasmid for the desired application
- *Bbs*I restriction enzyme (Thermo Scientific)
- T4 ligase buffer (NEB)
- T4 PNK enzyme (NEB)
- T7 ligase and buffer (Enzymatics)
- FastDigest Buffer (Thermo Scientific)
- FastAP enzyme (Thermo Scientific)

Protocol

1. Set up a restriction digest reaction as shown below and incubate at 37 °C for 30 min:
   1 μg pL018 (or other suitable plasmid)
   2 μl 10 × FastDigest Buffer
   1 μl FastAP
   1 μl *Bbs*I or other suitable enzyme
   Water to 20 μl total volume
2. Purify reaction products using a PCR purification kit and normalize concentration to 10 ng/μl.
3. Resuspend sense and antisense sgRNA oligos to 100 μ*M* and anneal using the following reaction mixture:
   1 μl 100 μ*M* sense sgRNA oligo
   1 μl 100 μ*M* antisense sgRNA oligo
   1 μl 10 × T4 ligation buffer
   0.5 μl T4 PNK (NEB)
   6.5 μl water
   Note: In this step, use T4 ligation buffer with the PNK enzyme as this contains ATP required for phosphorylation of the annealed oligos.

Use a thermocycler with the following program to phosphorylate and anneal oligos:

- 37 °C—30 min
- 95 °C—5 min
- Ramp from 95 °C to 25 °C at 5 °C/min

4. Dilute the annealed oligos from step 3 by 200-fold using water (to 50 n*M*). If the concentration is too high, multiple copies may be inserted into the vector.

5. Ligate annealed oligos into digested vector as follows:

1 μl digested plasmid (from step 2)
1 μl diluted annealed oligos (from step 4)
5 μl 2 × Quick Ligase Buffer
0.5 μl T7 ligase
2.5 μl water
Incubate at room temperature for 5 min.

Note: While 5 min of ligation is generally sufficient, longer incubation periods may be used to increase colony numbers if necessary.

When using new vectors, we recommend performing negative controls in parallel using the same conditions but omitting the annealed oligos from the ligation reaction.

6. Transform 2 μl of ligation product into chemically competent *E. coli* using standard procedures and spread on LB plates containing ampicillin. Incubate the plates at 37 °C overnight.

7. Culture and miniprep single colonies and sequence to confirm successful cloning. In general, we have very high success rates for screening single colonies although more can be tested if necessary.