Supplementary Methods
pCFD5 cloning protocol

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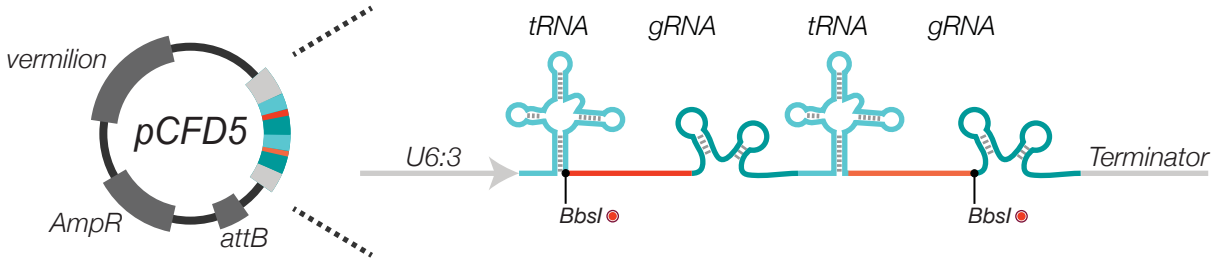
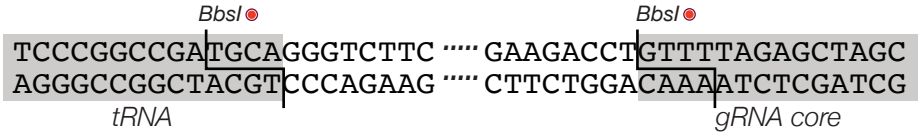


pCFD5 is a vector for expressing one or multiple tRNA-flanked Cas9 gRNAs under the control of the strong, ubiquitous RNA pol III promoter, U6:3.

Prepare the backbone:

Step 1: Digest the *pCFD5* plasmid with Bbs1 type-IIS restriction enzyme (NEB). We typically digest 8 µg DNA with 1 µl enzyme (10 u) and 3 µl 10x buffer in a 30 µl reaction for 2 - 4 h at 37°C.



The BbsI digest creates cohesive ends that allow seamless cloning of guide sequences.

Step 2: Run the digested plasmid on a 1% agarose gel. Cut out the backbone (9.6kb) and purify DNA using a commercial kit. Elute in 25 µl sterile dH_2O and measure DNA concentration. Concentration should be 50 - 200 ng/µl, which is enough for several cloning reactions.

Cloning one gRNA:

Cloning a single gRNA into the *pCFD5* plasmid uses annealed oligos, analogous to the cloning method used for *pCFD3* (Port et al. 2014). It is fast, cheap and highly efficient. Thus, many plasmids can be produced in parallel, if desired.

Step 1: Order standard desalted oligos without further purification:

Top oligo: 5′–TGCA(*guide sequence*)–3′

Bottom oligo: 5′–AAAC(*guide sequence (rev comp.)*)–3′

The guide sequence matches the genomic target site. The length of the guide sequence is usually 20 nt, but truncations are often tolerated and can make the CRISPR system more specific (Fu el al., 2014). Note that, whereas pCFD1 - 4 require a G at the first position of the guide sequence for optimal RNA pol III transcription, there is no such constraint for *pCFD5* as transcription is initiated before the 5' tRNA.

Step 2: Resuspend oligos in sterile dH_2O to a concentration of 100 µM.

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Cloning one gRNA (continued):

Step 3: Set up the following phosphorylation and annealing reaction:

1 µl	Top oligo (100 µM stock)
1 µl	Bottom oligo (100 µM stock)
1 µl	10x T4 Ligation Buffer (NEB)
6.5 µl	ddH_2O
0.5µl	T4 Polynucleotide Kinase (NEB)

Step 4: Incubate and anneal in a thermocycler:

37°C	30 min
95°C	5 min
ramp down to 20°C at 5°C/min	

Step 5: Set up the following ligation reaction:

X µl	BbsI digested *pCFD5* (60 ng)
1 µl	annealed oligos diluted 1:200 in ddH_2O
1.5 µl	10x T4 Ligation Buffer (NEB)
X µl	ddH_2O
1 µl	T4 DNA Ligase (NEB)

Total volume 15 µl

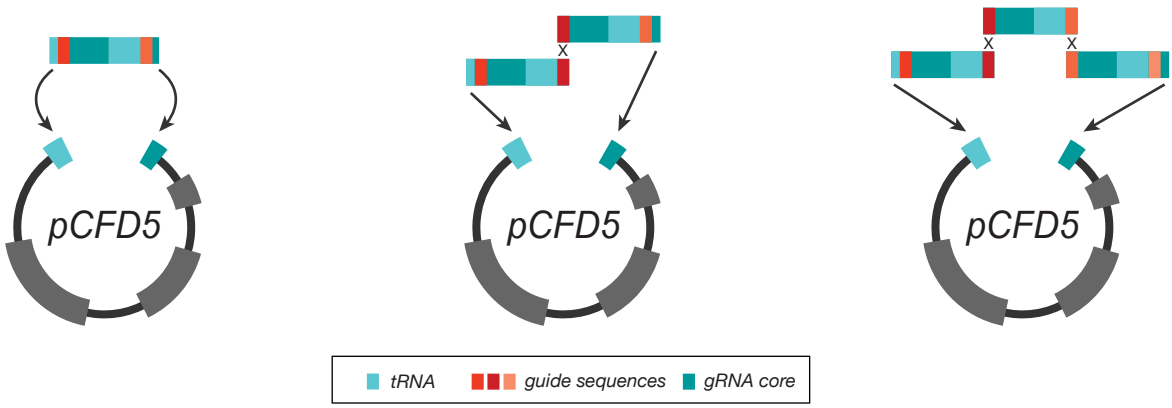
Step 6: Ligate 30 min at room temperature.

Step 7: Transform into competent bacteria. We use 2 µl of the ligation reaction to transform 70 µl chemically competent bacteria (Silver competent cells, Bioline). Let cells recover in 700 µl 2xTY media and plate out 100 µl on Ampicillin plates.

Step 8: Incubate plates at 37°C overnight.

Step 9: Pick two colonies and verify inserts by Sanger sequencing with primer U63seqfwd: 5'-ACGTTTTATAACTTATGCCCCTAAG-3'

Cloning 2 - 4 gRNAs:



The individual guide sequences are encoded in PCR primers that are used on a *pCFD5* template. The resulting PCR products are assembled with the linearised *pCFD5* backbone in a single Gibson Assembly reaction.

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Cloning 2 - 4 gRNAs (continued):

Below we illustrate how to clone four gRNAs from three overlapping PCR products. Two or one PCR products can be used to clone, respectively, three or two tRNA-flanked gRNAs. In all cases, the 5'- and 3'- most sequences of the set of inserts must include homology arms for Gibson Assembly into *pCFD5*.

Step 1: Order the oligos illustrated below (5'→3'):

Distal forward primer:

PCR1fwd:

guide sequence 1

GCGGCCCGGGTTCGATTCCCGGCCGATGCANNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG

Internal primers:

PCR1rev:

guide sequence 2 (rev comp.)

NNNNNNNNNNNNNNNNNNNNTGCACCAGCCGGGAATCGAACCC

PCR2fwd:

guide sequence 2

NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG

PCR2rev:

guide sequence 3 (rev comp.)

NNNNNNNNNNNNNNNNNNNNTGCACCAGCCGGGAATCGAACCC

PCR3fwd:

guide sequence 3

NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAG

Distal reverse primer:

PCR3rev:

guide sequence 4 (rev comp.)

ATTTTAACTTGCTATTTCTAGCTCTAAAACNNNNNNNNNNNNNNNNNNNTGCACCAGCCGGGAATCGAACCC

Distal primers include the homology arms for Gibson Assembly and are required regardless of the number of gRNAs cloned. Internal primers vary in number and introduce additional gRNAs.

We use standard desalted oligos. Although oligos of this length can contain some mistakes without further purification, we feel that the cheaper price and faster turn-around time more than compensates for the additional clones that have to be sequenced. As described above, the first position of the guide sequence can be any base and the length of the guide sequence is usually 20 nt. However, truncations to the guide sequence are often tolerated and can increase specificity of mutagenesis.

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Cloning of 2 - 4 gRNAs (continued):

Step 2: Run the individual PCRs with the primers above and *pCFD5* as template. Use a high-fidelity polymerase according to the manufacturer's guidelines. We use the 2x Q5 hot-start kit from NEB and an annealing temperature of 61°C that increases 0.5°C per cycle until a temperature of 72°C is reached (total number of cycles: 32).

Step 3: Run the PCR products on an 1% agarose gel. The expected sizes are PCR1, 234 bp; PCR2, 204 bp; PCR3, 234 bp. Cut out the correct bands and extract the DNA using a commercial kit.

Step 4: Prepare a Gibson Assembly reaction using ~50 ng of digested backbone and an ~two-fold molar excess of each insert. The typical reaction volume is 15 µl. Incubate for 1 h at 50°C.

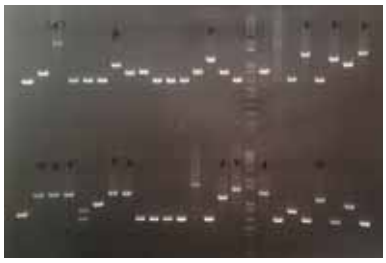
Step 5: Transform 2 µl of Gibson Assembly mix into 70 µl chemically competent cells using standard procedures (note that too high a proportion of Gibson Assembly mix interferes with transformation). Plate cells on Ampicillin plates and incubate at 37°C overnight.

Recommended:
The assembly of more than two fragments by Gibson Assembly is error prone. To reduce the number of clones that need to be sequenced in these cases, we perform colony PCR to select clones with inserts that appear to be of the right size.

Step 6: Pick single colonies with a pipet tip or plastic inoculation needle and dissolve in 10 µl bacterial culture medium containing Amplicillin. Use 1 µl of this medium as a template in a diagnostic PCR using an inexpensive PCR system (e.g. NEB Quick load Taq 2x master mix) and the following primers:

U63seqfwd: 5'-ACGTTTTATAACTTATGCCCCTAAG-3'

pCFDseqrev: 5'-GCACAATTGTCTAGAATGCATAC-3'



An example of a colony PCR from a Gibson Assembly of four gRNAs and *pCFD5*. Colonies marked by an asterisk had the approximate size predicted for the desired product and were analysed by sequencing.

Step 7: Examine PCR products on a 1% agarose gel. Colonies that give a product of the correct size (three gRNAs, 1013 bp; four gRNAs, 1186 bp) should be cultivated in 1 - 5 ml media overnight and miniprep DNA analysed by Sanger sequencing with primer U63seqfwd: 5'-ACGTTTTATAACTTATGCCCCTAAG-3' and pCFDseqrev 5'-GCACAATTGTCTAGAATGCATAC-3'.

Cloning of 5 - 6 gRNAs:

Cloning of more than four gRNAs follows the same logic as cloning three or four gRNAs. However, in our hands directly assembling more than four fragments with Gibson Assembly is inefficient. We therefore fuse individual PCR products to reduce the number of inserts to no more than three before using them in a Gibson Assembly reaction.

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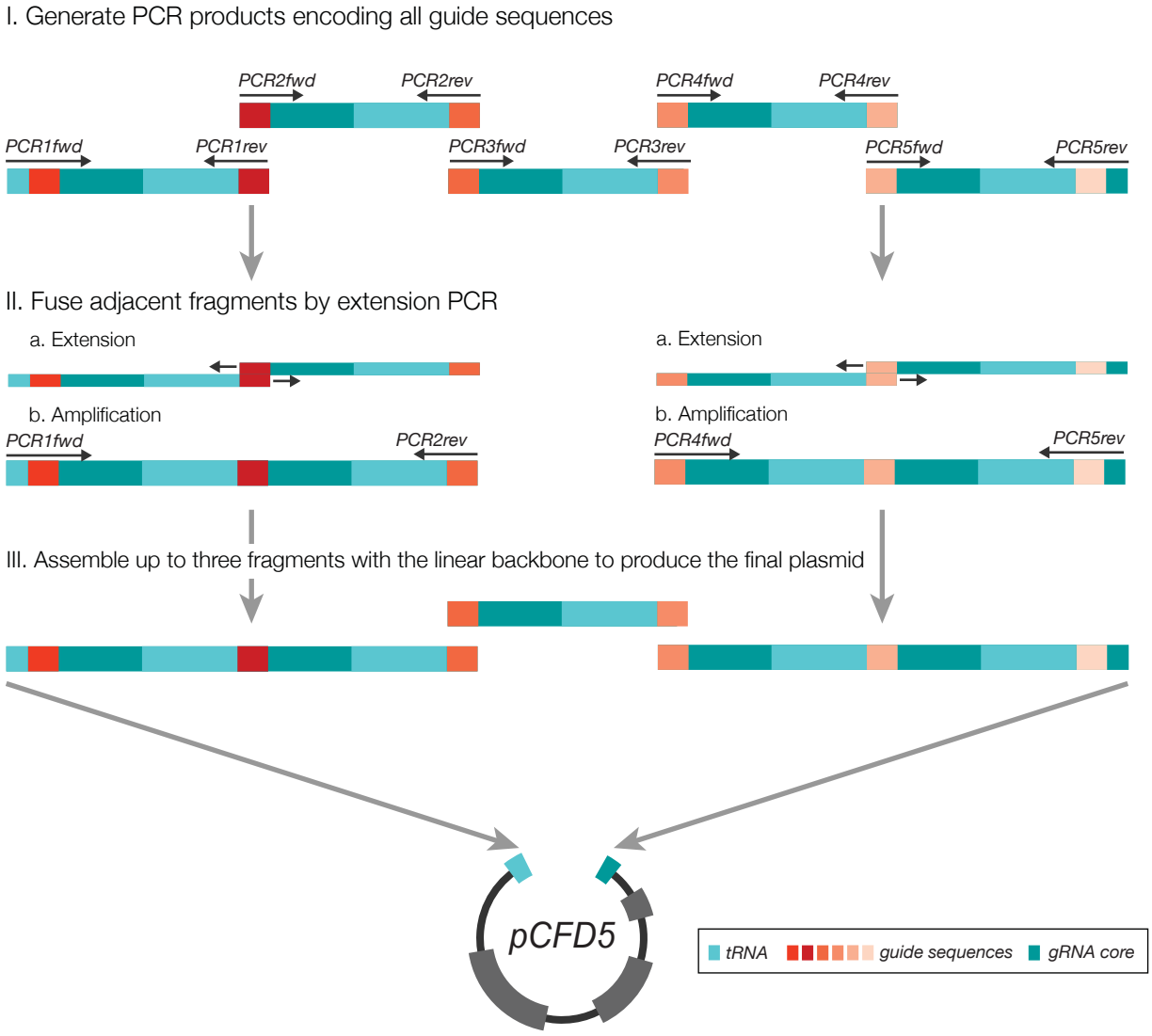
Cloning 5 - 6 gRNAs (continued):



Follow the protocol above for 2 - 4 gRNAs using additional internal primer pairs to generate 4 fragments (for 5 gRNAs) or 5 fragments (for six gRNAs).

Once the individual PCR products have been purified (Step 3), assemble two short adjacent fragments into one longer fragment. Use 300 ng of each PCR product in a PCR reaction mix without primers that has 15 cycles and an annealing temperature of 61°C. The overlapping ends of the two fragments will prime extension of each fragment. Add distal primers (e.g., PCR1fwd and PCR2rev) and run PCR for an additional 20 cycles with an annealing temperature of 61°C. Purify the desired product from an agarose gel. Once you have reduced the number of inserts to three, follow the protocol above for Gibson Assembly and identification of correct inserts. In Step 7, the expected product sizes for five and six gRNAs are 1360 bp and 1534 bp, respectively.

References:
Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol.* 2014 Mar;32(3):279-84. doi: 10.1038/nbt.2808. Epub 2014 Jan 26.

Port F, Chen HM, Lee T, Bullock SL. Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. Proc Natl Acad Sci U S A. 2014 Jul 22;111(29):E2967-76. doi: 10.1073/pnas.1405500111. Epub 2014 Jul 7.