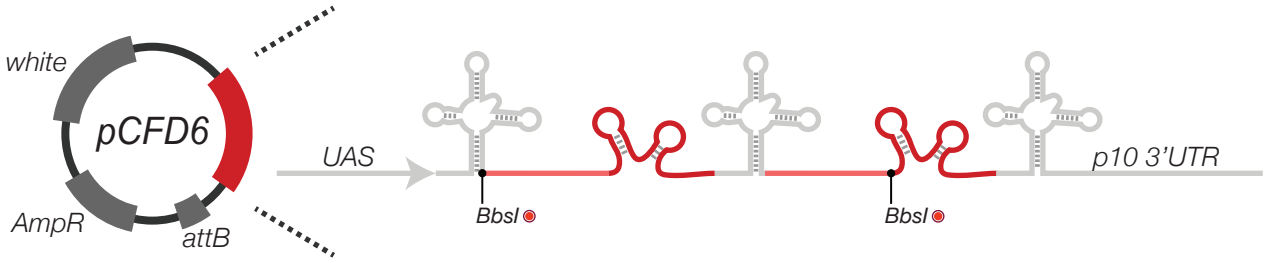
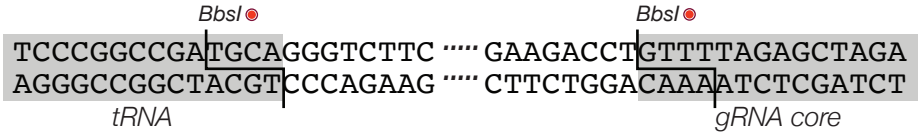
# pCFD6 cloning protocol




pCFD6 is a vector designed to express one or multiple gRNAs under of the Gal4/UAS system. Unlike most other pCFD vectors it contains a white transformation marker.

## Prepare the backbone:

Step 1: Digest the pCFD6 plasmid with the Bbs1 type-IIS restriction enzyme. We typically digest 8 µg DNA with 1 µl enzyme and 3 µl 10x buffer in a 30 µl reaction for 2-4 h at 37°C. Optional: Dephorphorylate the DNA ends with alkaline Phosphatase.




The BbsI digest creates sticky 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 spin column. Elute in 25 µl sterile dH2O and measure DNA concentration. You should have a concentration in the range of 50 -200 ng/µl, which is enough for several cloning reactions.

## Cloning of one gRNA:

Cloning a single gRNA into the pCFD6 plasmid uses annealed oligos, analogous to the cloning method used for pCFD3. It is fast, cheap and highly efficient and can be multiplexed if desired.

Step 1: Order the following oligos. We always use standard desalted oligos without further purification. The first position of the guide sequence (=your target site) can be any base. The length of the guide sequence is usually 20 nt, but truncations are often tolerated and can make the CRISPR system more specific (see Fu el al., 2014).

*guide sequence*

Top-oligo: TGCANNNNNNNNNNNNNNNNNNN

Bottom-oligo: AAACNNNNNNNNNNNNNNNNNNN

*guide sequence (rev comp.)*



continued...

## Cloning of 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 | Bbs1 digested pCFD6 (use 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

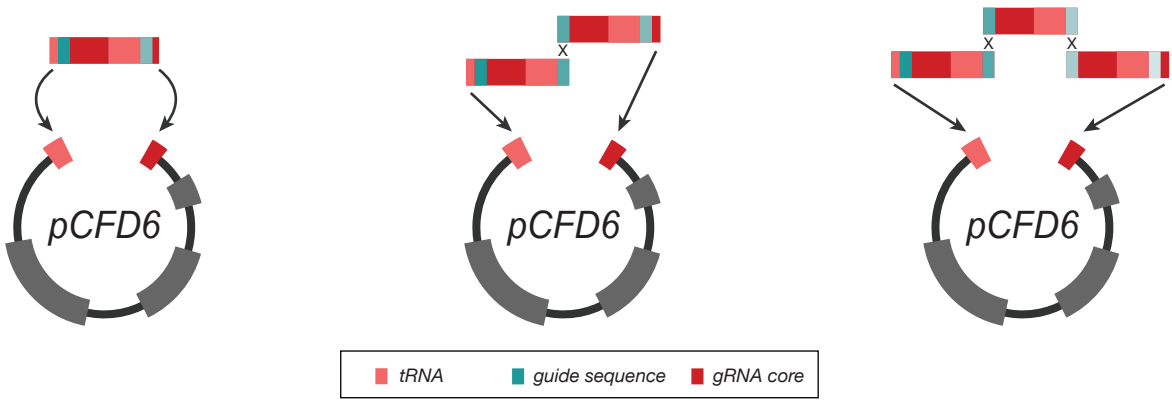
Step 6: Ligate 30 min at room temperature.

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

Step 8: Plate on Ampicillin plates and incubate at 37°C overnight.

Step 9: Pick two colonies and verify inserts by Sanger sequencing with primer pCFD6seqfwd: 5'-GTAGACATCAAGCATCGGTGG-3'

## Cloning of 2 - 4 gRNAs:




The individual guide sequences are encoded in PCR primers that are used on a *pCFD6* template. The resulting PCR products are assembled with the linearised *pCFD6* backbone in a single Gibson Assembly reaction.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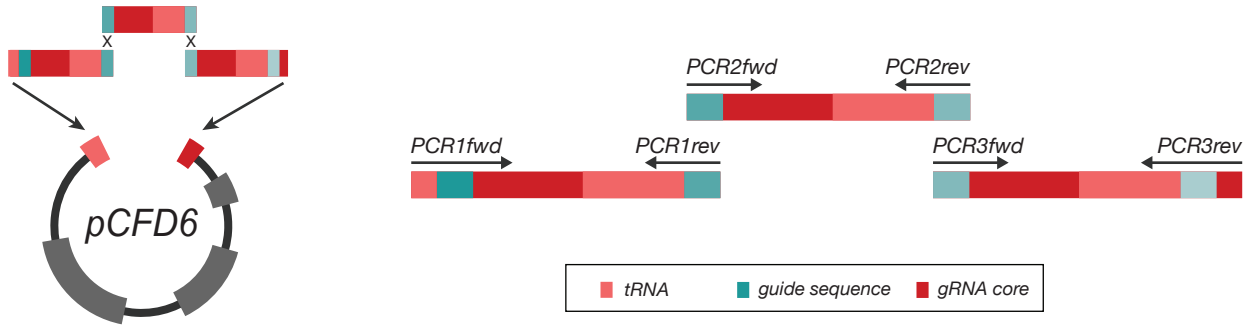
## Cloning of 2 - 4 gRNAs (continued):

Below is illustrated 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.




Step 1: Order the oligos illustrated below:

PCR1fwd:
*guide sequence 1*
CGGCCCGGGTTCGATTCCCGGCCGATGCANNNNNNNNNNNNNNNNNNNGTTTCAGAGCTATGCTGGAAAC

PCR1rev:
*guide sequence 2 (rev comp.)*
NNNNNNNNNNNNNNNNNNNNTGCACCAGCCGGGAATCGAACC

PCR2fwd:
*guide sequence 2*
NNNNNNNNNNNNNNNNNNNNGTTTCAGAGCTATGCTGGAAAC

PCR2rev:
*guide sequence 3 (rev comp.)*
NNNNNNNNNNNNNNNNNNNNTGCACCAGCCGGGAATCGAACC

PCR3fwd:
*guide sequence 3*
NNNNNNNNNNNNNNNNNNNNGTTTCAGAGCTATGCTGGAAAC

PCR3rev:
*guide sequence 4 (rev comp.)*
ATTTTAACTTGCTATTTCTAGCTCTAAAACNNNNNNNNNNNNNNNNNNNNTGCACCAGCCGGGAATCGAACC

Step 1: 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 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.

Step 2: Run the individual PCRs with the primers above and *pCFD6* 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 72°C (total number of cycles: 32).

Step 3: Run the PCR products on an 1% agarose gel. The expected sizes are PCR1, 233 bp; PCR2, 204 bp; PCR3, 234 bp. Cut out the correct bands and extract the DNA using a commercial kit.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## Cloning of 2 - 4 gRNAs (continued):

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.

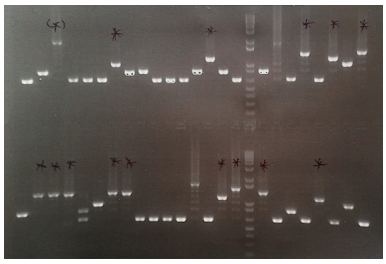
Step 5: Transform 2 µl of Gibson Assembly mix into 70 µl chemically competent cells using standard procedures (note that too high a proportion of Gibson Assembly mix interferes with transformation of competent cells). Plate cells on Ampicillin plates and incubate at 37°C overnight.

Recommended:
The assembly of > 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 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:

fwd: GTAGACATCAAGCATCGGTGG

rev: TTAGAGCTTTAAATCTCTGTAGGTAG



An example of a colony PCR from a Gibson Assembly of four gRNAs and *pCFD5* (*pCFD6* gives similar results). 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 an 1% agarose gel. Colonies that give 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 pCFD6seqfwd: 5'-GTAGACATCAAGCATCGGTGG-3'.

## Cloning of 5 - 6 gRNAs:

Cloning of more than four gRNAs follows the same logic as cloning three or four. 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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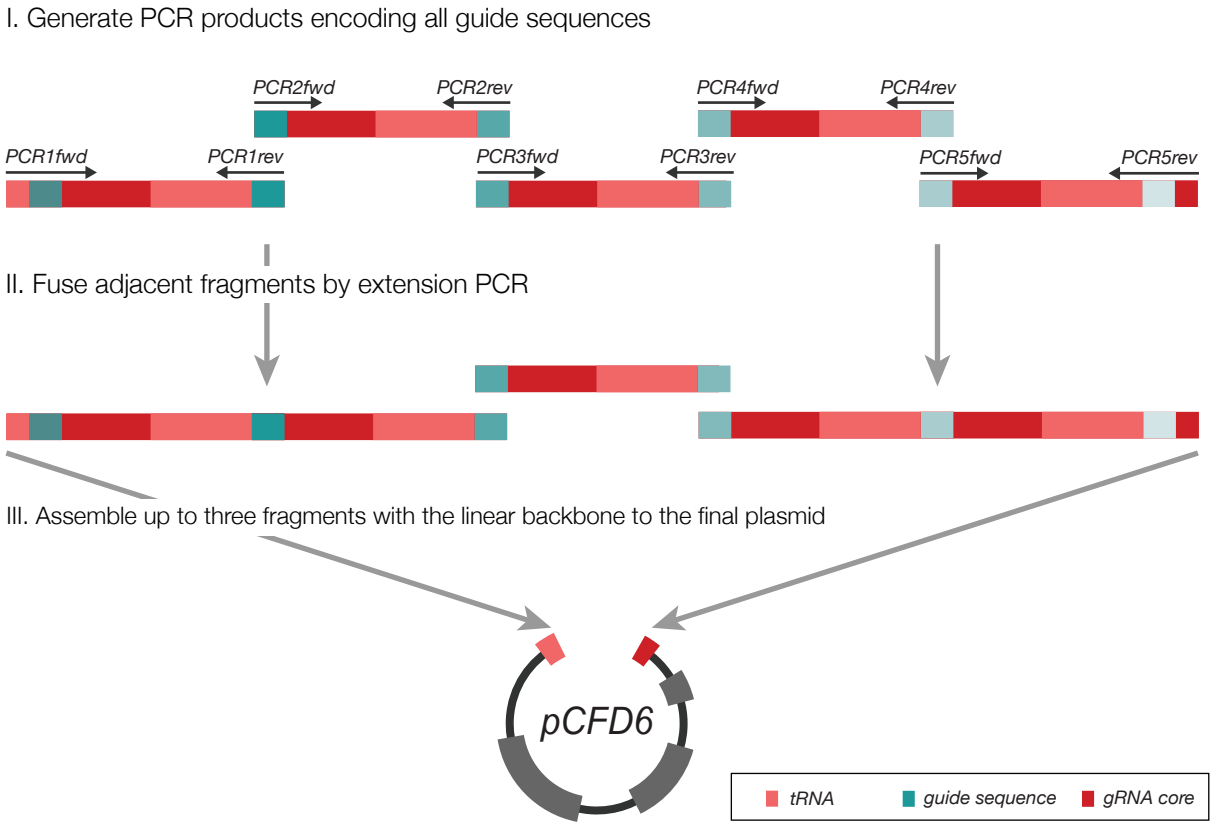
## Cloning of 5 - 6 gRNAs (continued):




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 you have purified the individual PCR products (Step 3), assemble two short adjacent fragments into one longer fragment. Use 300 ng of each PCR product in a PCR without primers that has 15 cycles with 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 for an additional 20 cycles with an annealing temperature of 61°C. 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.

Noticed a mistake in this protocol? Please contact Fillip at f.port@dkfz.de