PROTOCOL 3C

Assembly of multiple gRNA spacers into ribozyme arrays

Time needed to complete this protocol: **3 days (1 cloning step)**

Time needed to obtain the plant transformation vector with an gRNA array and Cas9: **6 days (2 cloning steps) – modular vectors, 3 days (1 cloning step) – DIRECT vectors**

Vectors compatible with this protocol: **pMOD_B2403 (any target vector listed in PROTOCOLS 3A and 3B can also accept ribozyme arrays)**

Summary: The gRNA spacers contained in overlapping PCR products will be cloned into the SapI sites of the pMOD/pDIRECT vector, replacing the ccdB gene. Correct pMOD clones are ready for assembly into transformation backbones (PROTOCOL 5). Correct pDIRECT clones are ready for plant transformation after sequencing. See also PROTOCOL 3 description and example.

Enzymes:

- **proof reading DNA polymerase**
- **SapI**
- **Esp3I, BsaI, AarI**
- **T7 DNA ligase + buffer**

1. Design primers for each gRNA being assembled as follows:

   RIBO-first
   TCGTCTCCxxxxxxxxxxxxTCCGGTGACAAAAGCCCTGC – replace red Xs with first 12 bases – reverse complement, of the first gRNA spacer being assembled

   REP
   TCGTCTCAxxxxxxxxxxxxGTTTTAGAGCTAGAAATAGC – replace red Xs with the last 12 bases of the first gRNA being assembled (also use this primer for every new gRNA from second to the last)

   RIBO
   TCGTCTCCxxxxxxxxxxxxTCCGGTGACAAAAGCACC – replace red Xs with the first 12 bases – in reverse complement of the second gRNA being assembled (also use this primer for every new gRNA from second to the second last)

   The primer design can also be done using the online primer design tool, which accepts the list of 20 bp gRNA spacer sequences in fasta format as input:
   http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/

   For an example of primer design for assembly of 6 gRNAs see file "**PROTOCOL 3** – example design"

Note 1: Primers shown here use Esp3I (recognition site highlighted in blue) as the type IIs restriction enzyme to create the spacer specific 4 bp overhangs. We strongly recommend using Esp3I as the default enzyme, but BsaI must be used when the PvUbi1 promoter (or other promoter containing Esp3I sites) is a part of the assembly and AarI must be used for all assemblies into pDIRECT_25H, pDIRECT_25I, pDIRECT_26H and pDIRECT_26I. Do not use AarI as the enzyme for assembling gRNA spacers into pMOD vectors. To use BsaI, replace the first 8 bases of each primer RIBO-first, REP and RIBO with 5'-NGGTCTCN-3'. To use AarI, replace the first 8 bases of each primer RIBO-first, REP and RIBO with 5'-NNNCACCTGCNNNN-3'.

Note 2: Make sure that no SapI and Esp3I (or BsaI or AarI, depending on which enzyme is used) recognition sites are present in any of the gRNA spacer sequences and that bases in position 9-12 are not identical in any two of the gRNA spacers being assembled. In addition, when cloning into modules B and C that will be assembled into a transformation backbone using **PROTOCOL 5**, gRNA spacers that contain AarI sites should be avoided. The same applies when AarI is used for assembly of gRNAs into pDIRECT_25H, pDIRECT_25I, pDIRECT_26H and pDIRECT_26I.

Note 3: For optimal efficiency, these primers can be used for assembly of up to 6 gRNAs. The cloning efficiency ranges from up to 100% of correctly assembled clones with 2-4 gRNAs to 60-80% of correctly assembled clones with 5-6 gRNAs. Assembly of more gRNAs in a single reaction is possible, but the cloning efficiency will be compromised.

2. Set up a PCR reaction for each gRNA cassette using a proofreading DNA polymerase (Q5 or Phusion polymerases are recommended). Use the target cloning vector as template (see Note 4 below) with following primer combinations. For sequences of the promoter specific primers and primer RIBO_term see the table below.

   Assembly of **2** gRNA spacers:

   reaction #1: promoter specific primer + RIBO-first_gRNA1
   reaction #2: REP_gRNA1 + RIBO_gRNA2
   reaction #3: REP_gRNA2 + RIBO_term

   Assembly of **3** gRNA spacers:

   reaction #1: promoter specific primer + RIBO-first_gRNA1
   reaction #2: REP_gRNA1 + RIBO_gRNA2
   reaction #3: REP_gRNA2 + RIBO_gRNA3
   reaction #4: REP_gRNA3 + RIBO_term

   Assembly of **4** gRNA spacers:

   reaction #1: promoter specific primer + RIBO-first_gRNA1
   reaction #2: REP_gRNA1 + RIBO_gRNA2
   reaction #3: REP_gRNA2 + RIBO_gRNA3

reaction #4: REP_gRNA3 + RIBO_gRNA4
reaction #5: REP_gRNA4 + RIBO_term

Assembly of **5** gRNA spacers:

reaction #1: promoter specific primer + RIBO-first_gRNA1
reaction #2: REP_gRNA1 + RIBO_gRNA2
reaction #3: REP_gRNA2 + RIBO_gRNA3
reaction #4: REP_gRNA3 + RIBO_gRNA4
reaction #5: REP_gRNA4 + RIBO_gRNA5
reaction #6: REP_gRNA5 + RIBO_term

Assembly of **6** gRNA spacers:

reaction #1: promoter specific primer + RIBO-first_gRNA1
reaction #2: REP_gRNA1 + RIBO_gRNA2
reaction #3: REP_gRNA2 + RIBO_gRNA3
reaction #4: REP_gRNA3 + RIBO_gRNA4
reaction #5: REP_gRNA4 + RIBO_gRNA5
reaction #6: REP_gRNA5 + RIBO_gRNA6
reaction #7: REP_gRNA6 + RIBO_term

Note 4: Most of the multi gRNA vectors (with the exception of pMOD_B2103b, pMOD_B2203 and pMOD_C2200) are cross-compatible and can accept cassettes amplified using any other vector as template (this is because all gRNA elements and the promoter are released from the vector during the Golden Gate cloning and are replaced with the elements present in the assembled PCR products). However, it is recommended to use the same vector as the template for PCR and cloning of the resulting PCR products. In case this is not possible (for example a ribozyme gRNA array is being assembled into a DIRECT vector but only a module B plasmid is available as template), it is recommended to use modular plasmids as templates and DIRECT vectors for cloning or vice versa. Different vectors of the same type (modular or DIRECT) should not be used as PCR template and cloning backbone, respectively. Unless the resulting PCR products are gel-purified before cloning, this might lead to a mixture of products with gRNA arrays inserted into both backbones.

3. Run the following cycle: 98°C/1min + 30x (98°C/10sec + 60°C/15sec + 72°C/15sec) + 72°C/2min + 4°C hold

4. Run 5 µl of each PCR product on a gel to verify that the amplification was successful. The first product will be longer (the size depends on the type of the amplified promoter), the second to the second last products will be +/- 129 bp and the last product will be +/- 164 bp. Gel purification is not essential and PCR products can be used directly (after dilution) in the Golden Gate reaction.

5. Dilute each PCR product 10 times.

6. Set up a Golden Gate reaction:
   a) 50 ng of the target vector
   b) 0.5 μl of each 10 times diluted PCR product (1-5 ng if gel-purified)
   c) 0.5 μl SapI (see Note 5)
   d) 0.5 μl Esp3I (or BsaI or AarI, depending on primer design, see Note 6)
   e) 1 μl T7 DNA ligase
   f) 10 μl 2x T7 DNA ligase buffer
   g) H_2O up to 20 μl

7. Mix the reaction by pipetting up and down several times

Note 5: SapI enzyme has to be mixed by pipetting up and down several times before adding to the reaction.

Note 6: AarI requires addition of a unique AarI oligonucleotide (supplied with the AarI enzyme). Add 0.4 μl per 20 μl Golden Gate reaction.

8. Place the reactions in the PCR machine and run cycle: 10x (37˚C/5min + 25˚C/10min) + 4°C hold

Note 7: Since the overhangs that type IIs restriction enzymes create between the gRNA cassettes are within the gRNA spacer sequences, they will be different in every set of gRNAs. Some combinations of overhangs might result in low cloning efficiency. If a correctly assembled clone cannot be obtained with a specific set of gRNAs, increasing the number of Golden Gate cycles to 20 will often help to increase the cloning efficiency.

9. Transform 5 μl (see Note 8) of the Golden Gate reaction into *E. coli* (DH5α or similar, but sensitive to the presence of ccdB gene) and plate on LB + 50mg/L of appropriate antibiotic - ampicillin/carbenicillin for modules B and C, kanamycin for DIRECT T-DNA vectors or spectinomycin for DIRECT non-T-DNA vectors.

Note 8: In some *E. coli* strains, the presence of PEG in the Golden Gate reaction (from the T7 ligase buffer) can have negative effect on transformation efficiency and using 2 μl of the Golden Gate reaction for transformation instead of 5 μl might significantly increase the number of obtained colonies. However, this is not true for all strains and the optimal transformation conditions have to be determined empirically.

10. Screen 5-10 colonies with primers specific for the type of vector and promoter used (see table below). If 2-3 gRNA spacers are cloned, screening 3-5 colonies should be sufficient to identify correct clones.

11. Isolate the plasmid DNA for one correct clone (can be sequenced, see the table below for sequencing primer).

12. gRNA arrays in modules B and C can be assembled with an appropriate Cas9 cassette in module A into selected transformation backbones using **PROTOCOL 5**.

Promoter specific primers for use in reaction #1 (5' to 3', SapI site is highlighted in blue and the 3 bp overhang is underlined):

promoter	primer
CmYLCV	TGCTCTTCGCGCTGGCAGACATACTGTCCCAC
35S	TGCTCTTCGCGCATGGAGTCAAAGATTCAAATAGAGG
YAO	TGCTCTTCGCGCGATGGGAAATTCATTGAAAACCC
PvUbi1	TGCTCTTCGCGCCACGTCAGTGTTTGGTTTCC

Last primer for use in the last reaction (5' to 3', SapI site is highlighted in blue and the 3 bp overhang is underlined):

RIBO_term	TGCTCTTCTGACGCTTTTGTTTCGTCCTCACG

Primers for colony PCR and sequencing – module B and C plasmids, DIRECT non-T-DNA plasmids (5' to 3')

promoter	primer name	primer sequence
CmYLCV	TC320	CTAGAAGTAGTCAAGGCGGC
	TC089R	GGAACCCTAATTCCCTTATCTGG
35S	ZY010F	ACGTAAGGGATGACGCACA
	TC089R	GGAACCCTAATTCCCTTATCTGG
YAO	TC429	TTCTTCCGTCGCCACTAAAC
	TC089R	GGAACCCTAATTCCCTTATCTGG
PvUbi1	TC306	AGCACTACCAATGATGACCT
	TC089R	GGAACCCTAATTCCCTTATCTGG

Primers for colony PCR and sequencing – DIRECT T-DNA plasmids (5' to 3')

promoter	primer name	primer sequence
CmYLCV	TC320	CTAGAAGTAGTCAAGGCGGC
	M13F	GTAAAACGACGGCCAGT
35S (only M13F primer can be used for sequencing)	ZY010F	ACGTAAGGGATGACGCACA
	M13F	GTAAAACGACGGCCAGT
YAO	TC429	TTCTTCCGTCGCCACTAAAC
	M13F	GTAAAACGACGGCCAGT
PvUbi1	TC306	AGCACTACCAATGATGACCT
	M13F	GTAAAACGACGGCCAGT