

PROTOCOL 3A

Assembly of multiple gRNA spacers into Csy4 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_B2101, pMOD_B2103, pMOD_B2103b (see step 2), pMOD_B2109, pMOD_B2112, pDIRECT_10C, pDIRECT_21C, pDIRECT_21D, pDIRECT_22C, pDIRECT_22D, pDIRECT_23C, pDIRECT_23D, pDIRECT_25H, pDIRECT_25I, pDIRECT_26H, pDIRECT_26I** (any target vector listed in PROTOCOLS 3B and 3C can also accept Csy4 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
- BanI
- SapI
- Esp3I, BsaI, AarI
- T7 DNA ligase + buffer

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

CSY

TCGTCTCxxxxxxxxxxxx**CTGCCTATACGGCAGTGAAC** – replace red Xs with first 12 bases – reverse complement, of the first gRNA spacer being assembled (also use this primer for every new gRNA from second to the second last)

REP

TCGTCTCxxxxxxxxxxxx**GTTTTAGAGCTAGAAATAGC** – 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)

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 CSY and REP with 5'-NGGTCTCN-3'. To use AarI, replace the first 8 bases of each primer CSY and REP and 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 or start with **GTG** (which creates AarI site when inserted immediately downstream of a Csy4 site) 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. For a more efficient protocol for assembly of 7-12 gRNAs using modules pMOD_B2203 and pMOD_C2200, see the supplementary **PROTOCOL 3S1**.

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 and 5 below) with following primer combinations. For sequences of the promoter specific primers and primer CSY_term see the table below. For assembly into pMOD_B2103b (only for Csy4 gRNA arrays), reaction #1 is skipped and primer REP_gRNA1 in reaction #2 is replaced with primer REP-full_gRNA1 (see **PROTOCOL 3S1**).

Assembly of 2 gRNA spacers:

reaction #1: promoter specific primer + CSY_gRNA1 (see Note 4)

reaction #2: REP_gRNA1 + CSY_gRNA2

reaction #3: REP_gRNA2 + CSY_term

Assembly of 3 gRNA spacers:

reaction #1: promoter specific primer + CSY_gRNA1 (see Note 4)

reaction #2: REP_gRNA1 + CSY_gRNA2

reaction #3: REP_gRNA2 + CSY_gRNA3

reaction #4: REP_gRNA3 + CSY_term

Assembly of 4 gRNA spacers:

reaction #1: promoter specific primer + CSY_gRNA1 (see Note 4)

reaction #2: REP_gRNA1 + CSY_gRNA2

reaction #3: REP_gRNA2 + CSY_gRNA3

reaction #4: REP_gRNA3 + CSY_gRNA4

reaction #5: REP_gRNA4 + CSY_term

Assembly of 5 gRNA spacers:

reaction #1: promoter specific primer + CSY_gRNA1 (see Note 4)

reaction #2: REP_gRNA1 + CSY_gRNA2

reaction #3: REP_gRNA2 + CSY_gRNA3

reaction #4: REP_gRNA3 + CSY_gRNA4

reaction #5: REP_gRNA4 + CSY_gRNA5

reaction #6: REP_gRNA5 + CSY_term

Assembly of 6 gRNA spacers:

reaction #1: promoter specific primer + CSY_gRNA1 (see Note 4)

reaction #2: REP_gRNA1 + CSY_gRNA2

reaction #3: REP_gRNA2 + CSY_gRNA3

reaction #4: REP_gRNA3 + CSY_gRNA4

reaction #5: REP_gRNA4 + CSY_gRNA5

reaction #6: REP_gRNA5 + CSY_gRNA6

reaction #7: REP_gRNA6 + CSY_term

Note 4: Due to the presence of two Csy4 repeats in each template vector two products can be amplified in the **reaction #1** (when amplifying the promoter containing fragment) since the reverse primer binds to the repeated sequence. This can be prevented by using BanI digested plasmid as the template. BanI cleaves the gRNA repeat sequence and separates the two Csy4 repeats. Alternatively, the shorter, desired product can be gel-purified when non-digested plasmid is used as a template. The presence of these repeats does not affect reactions #2-#7 and therefore gel purification is not necessary and BanI digested plasmid must NOT be used in reactions #2-#7.

Note 5: 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 a case when 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. Do not do extra PCR cycles to obtain more product.
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), all the other products will be +/- 136 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 6)
 - d) 0.5 µl Esp3I (or BsaI or AarI, depending on primer design, see Note 7)
 - e) 1 µl T7 DNA ligase
 - f) 10 µl 2x T7 DNA ligase buffer
 - g) H₂O up to 20 µl
7. Mix the reaction by pipetting up and down several times

Note 6: SapI enzyme has to be mixed by pipetting up and down several times before adding to the reaction.

Note 7: 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 9: Since the overhangs that type II 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 10) 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 10: 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	<u>TGCTCTTC</u> GCGCTGGCAGACATACTGTCCCAC
35S	<u>TGCTCTTC</u> GCGCATGGAGTCAAAGATTCAAATAGAGG
YAO	<u>TGCTCTTC</u> GCGCGATGGGAAATTCATTGAAAACCC
PvUbi1	<u>TGCTCTTC</u> GCGCCACGTCAGTGTTTGTTTCC

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

CSY_term	<u>TGCTCTTC</u> TGACCTGCCTATACGGCAGTGAAC
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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	GGAACCCTAATTCCTTATCTGG
35S	ZY010F	ACGTAAGGGATGACGCACA
	TC089R	GGAACCCTAATTCCTTATCTGG
YAO	TC429	TTCTTCCGTCGCCACTAAAC
	TC089R	GGAACCCTAATTCCTTATCTGG
PvUbi1	TC306	AGCACTACCAATGATGACCT
	TC089R	GGAACCCTAATTCCTTATCTGG

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