PROTOCOL 3S1

Supplementary protocol for assembly of 7 and more gRNA spacers into a Csy4 array

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

Time needed to obtain the plant transformation vector with a gRNA array and Cas9: **6 days (2 cloning steps)**

Vectors compatible with this protocol: **pMOD_B2203, pMOD_C2200**

Summary: The first 1-6 gRNA spacers contained in overlapping PCR products will be cloned into the SapI sites of pMOD_B2203 and the next 1-6 gRNA spacers will be cloned into the SapI sites of pMOD_C2200 vector, replacing the ccdB gene. Correct pMOD_B and pMOD_C clones are then combined in a transformation backbone (PROTOCOL 5) to create an array of up to 12 gRNA spacers. 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
   TCGTCTCCxxxxxxxxxxxxCTGCCTATACGGCAGTGAAC – replace red Xs with first 12 bases – reverse complement, of the first gRNA spacer being assembled (also use this primer for every new gRNA spacer from second to the second last)

   REP
   TCGTCTCAxxxxxxxxxxxxGTTTTAGAGCTAGAAATAGC – replace red Xs with the last 12 bases of the first gRNA spacer being assembled (also use this primer for every new gRNA spacer from second to the last, with the exception of the 7th gRNA spacer in the array)

   REP-full
   TGCTCTTCTCAGxxxxxxxxxxxxxxxxxxxxGTTTTAGAGCTAGAAATAGC – replace red Xs with 20 bases of the 7th gRNA spacer being assembled

   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 CSY and REP 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. To use BsaI, replace the first 8 bases of each primer CSY and REP with 5'-NGGTCTCN-3'.

Note 2: Make sure that no AarI, SapI, Esp3I (or BsaI 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. gRNA spacers that start with **GTG** (which creates AarI site when inserted immediately downstream of a Csy4 site) should also be avoided.

Note 3: For optimal efficiency, these primers can be used for assembly of up to 6 gRNAs into each pMOD_B2203 and pMOD_C2200. The cloning efficiency is ~60-80% of correctly assembled clones with 6 gRNAs. In order to construct expression vectors with more than 12 gRNAs, assembly of more gRNAs into each pMOD_B2203 and pMOD_C2200 vector 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 **pMOD_B2203** as template (see Note 4 below) with following primer combinations. For sequences of the promoter specific primers and primer CSY_term see the table below.

   Assembly of **7** 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
   reaction #8: REP_full_gRNA7 + CSY_term

   Assembly of **8** 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
   reaction #8: REP-full_gRNA7 + CSY_gRNA8
   reaction #9: REP_gRNA8 + CSY_term

Assembly of **9** 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
reaction #8: REP-full_gRNA7 + CSY_gRNA8
reaction #9: REP_gRNA8 + CSY_gRNA9
reaction #10: REP_gRNA9 + CSY_term

Assembly of **10** 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
reaction #8: REP-full_gRNA7 + CSY_gRNA8
reaction #9: REP_gRNA8 + CSY_gRNA9
reaction #10: REP_gRNA9 + CSY_gRNA10
reaction #11: REP_gRNA10 + CSY_term

Assembly of **11** 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
reaction #8: REP-full_gRNA7 + CSY_gRNA8
reaction #9: REP_gRNA8 + CSY_gRNA9
reaction #10: REP_gRNA9 + CSY_gRNA10
reaction #11: REP_gRNA10 + CSY_gRNA11
reaction #12: REP_gRNA11 + CSY_term

Assembly of **12** gRNA spacers:

reaction #1: promoter specific primer + oCsy4-B_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
reaction #8: REP-full_gRNA7 + CSY_gRNA8
reaction #9: REP_gRNA8 + CSY_gRNA9
reaction #10: REP_gRNA9 + CSY_gRNA10
reaction #11: REP_gRNA10 + CSY_gRNA11
reaction #12: REP_gRNA11 + CSY_gRNA12
reaction #13: REP_gRNA12 + 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-#13 and therefore gel purification is not necessary and BanI digested plasmid must NOT be used in reactions #2-#13.

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 (except from reaction #8) will be +/- 136 bp. Product from reaction #8 will be +/- 148 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. Setup two Golden Gate reactions:

   1st Golden Gate reaction:
   a) 50 ng of pMOD_B2203
   b) 0.5 μl of each 10 times diluted PCR product from reaction #1-#7 (1-5 ng if gel-purified)
   c) 0.5 μl SapI (see Note 5)
   d) 0.5 μl Esp3I
   e) 1 μl T7 DNA ligase
   f) 10 μl 2x T7 DNA ligase buffer
   g) H_2O up to 20 μl

2nd Golden Gate reaction:

a) 50 ng of pMOD_C2200
b) 0.5 μl of each 10 times diluted PCR product from reaction #8-#13 (1-5 ng if gel-purified)
c) 0.5 μl SapI (see Note 5)
d) 0.5 μl Esp3I
e) 1 μl T7 DNA ligase
f) 10 μl 2x T7 DNA ligase buffer
g) H_2O up to 20 μl

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

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

Note 6: 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.

8. Transform 5 μl (see Note 7) 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 ampicillin/carbenicillin.

Note 7: 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.

9. Screen 5-10 colonies with primers specific for pMOD_B2203 and pMOD_C2200 (see table below).

10. Isolate the plasmid DNA for one correct clone of each plasmid (can be sequenced, see the table below for sequencing primers).

11. Assemble gRNA arrays in modules B and C along with a Csy4-Cas9 cassette in module A into a selected transformation backbone 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):

CSY_term	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

Primers for colony PCR and sequencing – pMOD_B2203 (5' to 3')

promoter	primer name	primer sequence
CmYLCV	TC320	CTAGAAGTAGTCAAGGCGGC
	ZY015F	GGAATAAGGGCGACACGGAAATG
35S	ZY010F	ACGTAAGGGATGACGCACA
	ZY015F	GGAATAAGGGCGACACGGAAATG
YAO	TC429	TTCTTCCGTCGCCACTAAAC
	ZY015F	GGAATAAGGGCGACACGGAAATG
PvUbi1	TC306	AGCACTACCAATGATGACCT
	ZY015F	GGAATAAGGGCGACACGGAAATG

Primers for colony PCR and sequencing – pMOD_C2200 (5' to 3')

primer name	primer sequence
CS433	CTCACATGTTCTTTCCTGCG
TC089R	GGAACCCTAATTCCCTTATCTGG