PROTOCOL 5

Assembly of A, B and C modules into transformation backbones

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

Time needed to obtain the plant transformation vector: **3 days (1 cloning step)**

Vectors compatible with this protocol: **all module A, B and C plasmids and all transformation backbones**

Summary: The functional elements in the intermediate module plasmids A, B and C will be assembled into the AarI sites of the transformation backbone, replacing the ccdB gene. Correct clones are ready for plant transformation. See also PROTOCOL 5 description.

Enzymes:

- **AarI**
- **T4 DNA ligase**

1. Select one plasmid from each module A, B and C sets plus one transformation backbone – any combination of A, B and C plasmids is possible (for example module A with a Cas9 expression cassette, module B with gRNA spacers cloned in following **PROTOCOL 2** or **3** and module C with a donor template cloned in following **PROTOCOL 4**). One and only one plasmid of each module type is required for the reaction.

   Our online vector selection tool can be used to identify the appropriate plasmids according to users' specifications.

   http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/vector.php

2. Set up a Golden Gate reaction:

   a) 75 ng transformation backbone
   b) 150 ng module A plasmid
   c) 150 ng module B plasmid
   d) 150 ng module C plasmid
   e) 0.4 µl AarI oligonucleotide (comes with the AarI enzyme)
   f) 0.5 µl AarI
   g) 1 µl T4 DNA ligase
   h) 2 µl 10x T4 DNA ligase buffer
   i) H_2O up to 20 µl

3. Place the reactions in the PCR machine and run cycle: 10x (37˚C/5min + 16˚C/10min) + 37°C/15min + 80°C/5min + 4°C hold

Note 1: Although none of the plasmids contain additional AarI sites that would interfere with Golden Gate assembly, some GT donor template sequences might contain AarI sites. When using such donor sequences cloned in a module C plasmid, the last digestion step at 37°C/15min can be skipped to prevent digestion of the final product at the AarI site in the donor sequence which is not removed in the assembly process, unlike the AarI sites that flank the A, B and C cassettes. Correct clones can be obtained using this approach with moderate frequency, despite the presence of an intact AarI site in the final product. Alternatively, AarI sites can be easily removed during the donor assembly process (see **PROTOCOL 4**).

4. Transform 5 μl 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 - kanamycin for T-DNA vectors or spectinomycin for non-T-DNA vectors.

5. Optionally, correct clones can be identified by PCR on 2-3 colonies, using one of the reverse primers listed below with a forward primer specific to one of the cloned elements. For example, for vectors containing TALEN or Cas9 expression cassettes (depending on the size of the module B and C cassettes), forward primer TC430 which binds to the HSP terminator (see the table below for sequence) can be used. However, ~99% of clones are usually correct and colony PCR might not be necessary.

6. Isolate the plasmid DNA for one correct clone. The Golden Gate junctions can be sequenced, although the fidelity of cloning is usually high. Alternatively, the structure of the final plasmid can be verified by restriction digest analysis.

Forward primer for colony PCR and sequencing (5' to 3'):

TC430	GTTGGATCTCTTCTGCAGCA

This primer binds to the HSP terminator present in most Cas9 and GFP expression cassettes in module A plasmids and the 2nd TALEN expression cassettes in module B plasmids. It can only be used if one of these module A/B plasmids was used in the assembly. In all other cases, a new primer must be designed based on the sequence of the assembled modules.

Reverse primers for colony PCR and sequencing (5' to 3'):

M13F (for standard T-DNA vectors except pTRANS_200 and pTRANS_240)	GTAAAACGACGGCCAGT
TC211F (for pTRANS_200)	AACACATTGCGGACGTTTTT
oCS823 (for pTRANS_240)	AGTAACATAGATGACACCGGAGTG
NB442 (for all BeYDV vectors)	GCAATCCTGACGAAGACTGGATGT
TC077R2 (for all ToLCV vectors)	AATCCTGTTGCCGGTCTT
TC503 (for all WDV vectors)	GGTTTGTCTTTGCTCGTAGC
pCR8R1 (for standard non-T-DNA vectors)	CGAACCGAACAGGCTTATGT