PROTOCOL 4

Assembly of DNA donor templates for gene targeting into module C vectors

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

Time needed to obtain the plant transformation vector with a donor template and SSN expression cassette: **6 days (2 cloning steps) or 9 days (3 cloning steps) if gRNA spacers are cloned in module C**

Vectors compatible with this protocol: **all module C plasmids**

Summary: The donor template for gene targeting will be assembled from one or more PCR fragments into the BaeI site of the pMOD_C vector by Gibson assembly. Correct clones are ready for assembly into transformation backbones (PROTOCOL 5). See also PROTOCOL 4 description.

Enzymes:

- **proof reading DNA polymerase**
- **BaeI**
- **1.33x Gibson assembly master mix (make as 15µl aliquots, store at -20°C):**
  - **2 µl T5 exonuclease (1 U/µl)**
  - **6.25 µl Phusion DNA polymerase (2 U/µl)**
  - **50 µl Taq DNA ligase (40 U/µl)**
  - **100 µl 5x isothermal buffer (25% PEG-8000, 500 mM Tris-HCl pH7.5, 50 mM $MgCl_2$, 50 mM DTT, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 5 mM NAD)**
  - **H_2O to 375 µl**

1. Design primers as follows:

   oBaeI_FWD (sequence in blue overlaps with BaeI digested module C vector)
   CGCGTAGTCCTCGGTAxxxxxxxxxxxxxxxxxxxx – replace red Xs with a primer binding to the 5' end of the left homology arm

   Reverse primer (REV1) to be used with oBaeI_FWD that overlaps with the forward primer (FWD2) for the next fragment (genomic or insertion sequence). First 8 bp of the FWD2 primer is added to the 5' end of the REV1 primer in reverse complement and vice versa to create a 16 bp overlap. T_m of the overlap should be at least 48°C (add 2°C for each A-T pair and 4°C for each G-C pair to calculate the T_m). If T_m is below 48°C, the overlap can be extended until the T_m is at least 48°C. Sequence modifications (base substitutions for allele replacement or AarI removal) can be in the overlapping region of both primers. Additional primers are designed in the same way for each other fragment (e.g. REV2 pairing with FWD2 and overlapping with FWD3, REV3 pairing with FWD3 and overlapping with FWD4, etc.). Last FWD primer will pair with oBaeI_REV.

oBaeI_REV (sequence in blue overlaps with BaeI digested module C vector)
TGACTTGAAGTACACTCxxxxxxxxxxxxxxxxxxx – replace red Xs with a primer (reverse) binding to the 3' end of the right homology arm

2. Digest Module C vector to prepare it for Gibson Assembly:
   a) 2 μg of the target module C vector
   b) 1x restriction buffer
   c) 1 μl BaeI
   d) H_2O up to 20 μl

3. Incubate 1 hour at 25°C

4. Gel purify the linearized backbone and and measure the DNA concentration. (the BaeI cut plasmid can be stored at -20°C and used multiple times)

5. PCR-amplify parts of the donor template using the primers designed in step 1. Primer oBaeI_FWD pairs with REV1, FWD2 with REV2 etc. oBaeI_REV pairs with the last FWD primer.

6. Gel-purify the fragments of correct size and measure the DNA concentration.

7. Setup a Gibson assembly reaction:
   a) 15 μl of 1.33x Gibson master mix
   b) 50 ng of the BaeI digested module C vector
   c) equimolar amounts of each donor fragment, each in 3 times higher molar concentration than the module C backbone
   d) H_2O up to 20 μl

8. Incubate 1 hour at 50°C

9. Transform 5 μl of the reaction into *E. coli* (DH5α or similar) and plate on LB + 50mg/L ampicillin/carbenicillin.

10. Correct clones can be identified by colony PCR using primer ZY015F (see below) along with a primer specific to the donor sequence.

11. Isolate the plasmid DNA for one correct clone (can be sequenced with ZY015F and donor specific primers).

12. Assemble module C plasmid containing the donor template along with selected modules A and B into selected transformation backbone using **PROTOCOL 5**.

Primer for colony PCR and sequencing (5’ to 3’)

ZY015F	GGAATAAGGGCGACACGGAAATG