PROTOCOL 2A

Assembly of a single gRNA spacer into module B and C vectors

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

Time needed to obtain the plant transformation vector with a complete CRISPR/Cas9 reagent: **6 days (2 cloning steps)**

Vectors compatible with this protocol: **pMOD_B2515, pMOD_B2517, pMOD_B2518, pMOD_B2519, pMOD_B2520, pMOD_B2615, pMOD_C2515, pMOD_C2516, pMOD_C2517, pMOD_C2518, pMOD_C2519, pMOD_C2520, pMOD_C2616**

Summary: The gRNA spacer in the form of annealed oligos will be cloned into the Esp3I (or BsaI) sites of the pMOD vector, replacing the ccdB gene. Correct clones are ready for assembly into transformation backbones (PROTOCOL 5). See also PROTOCOL 2 description.

Enzymes:

- **Esp3I**
- **T4 DNA ligase**
- **T4 polynucleotide kinase + T4 DNA ligase buffer (contains ATP)**
- **DNA polymerase (for colony PCR)**

1. Synthesize two oligonucleotides containing the gRNA spacer sequence (usually 20 bp) and 4 bp overhangs specific for the type of Pol III promoter, as shown below. Replace the X symbols with the gRNA spacer sequence and Y symbols with sequence complementary to X. Also see the table below for oligonucleotide sequences in 5'-3' orientation. Note that sequences transcribed from promoters AtU6, At7SL, TaU6 and OsU6 must have a G nucleotide in the first position, while for OsU3 and TaU3 promoters it is an A nucleotide. These nucleotides are not included in the vector backbone and should be therefore included in the oligonucleotides.

AtU6 promoter:

```
GATTXXXXXXXXXXXXXXXXXXX
     ||||||||||||||||||||
     YYYYYYYYYYYYYYYYYYYCAAA
```

At7SL promoter:

```
GTACXXXXXXXXXXXXXXXXXXX
     ||||||||||||||||||||
     YYYYYYYYYYYYYYYYYYYCAAA
```

TaU3 promoter:

```
AAGCXXXXXXXXXXXXXXXXXXX
     ||||||||||||||||||||
     YYYYYYYYYYYYYYYYYYYCAAA
```

TaU6 promoter:

```
ACTTXXXXXXXXXXXXXXXXXXX
     ||||||||||||||||||||
     YYYYYYYYYYYYYYYYYYYCAAA
```

OsU3 promoter:

```
TGGCXXXXXXXXXXXXXXXXXXX
    ||||||||||||||||||||
   YYYYYYYYYYYYYYYYYYYYCAAA
```

OsU6 promoter:

```
TTGTXXXXXXXXXXXXXXXXXXXX
    ||||||||||||||||||||
   YYYYYYYYYYYYYYYYYYYYCAAA
```

2. Phosphorylate the oligonucleotides. (Alternatively, phosphorylated oligos may be purchased. Phosphorylating them yourself is efficient enough and much more cost effective.) Prepare the following reaction:

    a) 3 μl 100 μM sense gRNA oligonucleotide
    b) 3 μl 100 μM antisense gRNA oligonucleotide
    c) 3 μl T4 DNA ligase buffer (contains ATP)
    d) 2 μl T4 polynucleotide kinase
    e) 19 μl H_2O

3. Incubate 1 hour at 37°C.

4. Denature and gradually cool down the DNA. This can be done in a PCR machine using the following program: 95°C/5 min + ramping down to 85°C at -2°C/second + ramping down to 25°C at -0.1°C/second + 4°C hold **OR** by boiling the reaction in a water bath for 2 minutes and lettting it cool down gradually.

5. Dilute the reaction 25 times (1 μl oligo mixture + 24 μl H_2O)

6. Setup a Golden Gate reaction:
    a) 50 ng of selected module B or C plasmid
    b) 1 μl 25x diluted annealed oligonucleotides
    c) 0.5 μl Esp3I (select modules use BsaI instead, check the plasmid description and/or map)
    d) 2 μl 10X T4 DNA ligase buffer
    e) 1 μl T4 DNA ligase
    f) H_2O up to 20 μl

7. Place the Golden Gate reaction in a PCR machine and run the following cycle: 37˚C/5min + 16˚C/10min + 37˚C/15min + 80˚C/5min.

8. 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 ampicillin/carbenicillin.

9. Correct clones can be identified via colony PCR using the sense gRNA oligonucleotide as the forward primer and primer ZY015F (see the table below for primer sequence) as the reverse primer. However, this is usually not necessary thanks to the high cloning efficiency.

10. Isolate the plasmid DNA for one correct clone (can be sequenced using the ZY015F primer). Note that module C plasmids can be reused in another cloning step to insert a gene targeting donor following **PROTOCOL 4**.

11. Assemble gRNAs in modules B and/or C along with a Cas9 cassette in module A into selected transformation backbone using **PROTOCOL 5**.

Oligonucleotides in 5' to 3' orientation

AtU6 sense gRNA oligo	GATTXXXXXXXXXXXXXXXXXX
At7SL sense gRNA oligo	GTACXXXXXXXXXXXXXXXXXX
TaU3 sense gRNA oligo	AAGCXXXXXXXXXXXXXXXXXX
TaU6 sense gRNA oligo	ACTTXXXXXXXXXXXXXXXXXX
OsU3 sense gRNA oligo	TGGCXXXXXXXXXXXXXXXXXX
OsU6 sense gRNA oligo	TTGTXXXXXXXXXXXXXXXXXX
antisense gRNA oligo (all)	AAACYYYYYYYYYYYYYYYYY
ZY015F	GGAATAAGGGCGACACGGAAATG