PROTOCOL 2B

Assembly of a single gRNA spacer into DIRECT 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: **3 days (1 cloning step)**

Vectors compatible with this protocol: **pDIRECT_10A, pDIRECT_21A, pDIRECT_21B, pDIRECT_22A, pDIRECT_22B, pDIRECT_23A, pDIRECT_23B, pDIRECT_25F, pDIRECT_25G, pDIRECT_26F, pDIRECT_26G**

Summary: The gRNA spacer in the form of annealed oligos will be cloned into the AarI sites of the pDIRECT vector, replacing the LacZ gene. Correct clones are ready for plant transformation after sequencing. See also PROTOCOL 2 description.

Enzymes:

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

Follow steps 1.-5. of **PROTOCOL 2A**.

6. Setup a Golden Gate reaction:

    a) 50 ng of selected DIRECT plasmid
    b) 1 µl 25x diluted annealed oligonucleotides
    c) 0.4 µl AarI oligonucleotide (comes with the AarI enzyme)
    d) 0.5 µl AarI
    e) 2 µl 10X T4 DNA ligase buffer
    f) 1 µl T4 DNA ligase
    g) 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) and plate on LB + 50mg/L kanamycin (for T-DNA vectors) or spectinomycin (for non-T-DNA vectors) + 32mg/L X-gal.

9. Correct clones can be identified via PCR on white colonies using the sense gRNA oligonucleotide as the forward primer and primer NB463 (for T-DNA vectors) or pCR8R1 (for non-T-DNA vectors) as the reverse primer (see the table below for primer sequence). However, this is usually not necessary as due to the high cloning efficiency.

10. Isolate the plasmid DNA for one correct clone (can be sequenced using the NB463 or pCR8R1 primer).

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
NB463	CGAACGGATAAACCTTTTCACG
pCR8R1	CGAACCGAACAGGCTTATGT