Protocol for one sgRNA cloning

Simplified protocol

1. Manually search for 23-bp target sites (5'-N_{20}NGG-3') within exons of genomic DNA sequences of genes of interest, and then evaluate target specificities on the website of potential off-target finder (http://www.rgenome.net/cas-offinder/). Users can also search for target sites on the website of genome-wide prediction of plant CRISPR/Cas9 target sites (http://www.genome.arizona.edu/crispr/CRISPRsearch.html).

2. Design oligos/primers according to the sequences as follows:
   a) Oligo-01-F: 5'-ATTGNNNNNNNNNNNNNNN
   b) Oligo-R: 5’-AAACNNNNNNNNNNNNNNNNN
   (The oligos require no phosphorylation. Just submit the 23-nt oligos for synthesis as common primers)

3. To generate an insert, mix the two oligos (100 μmol/L each), heat at 95°C for 5 min, and cool down to room temperature.

4. Set up Golden Gate reactions as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert (0.5~50 μmol/L)</td>
<td>2 μl</td>
<td>5 hours at 37°C</td>
</tr>
<tr>
<td>pHHE401E (~100 ng/μl)</td>
<td>2 μl</td>
<td>5 min at 50°C</td>
</tr>
<tr>
<td>10× T4 DNA Ligase Buffer (NEB)</td>
<td>1.5 μl</td>
<td>10 min at 80°C</td>
</tr>
<tr>
<td>10× BSA</td>
<td>1.5 μl</td>
<td></td>
</tr>
<tr>
<td>BsaI (NEB)</td>
<td>1 μl</td>
<td></td>
</tr>
<tr>
<td>T4 DNA Ligase (HC, NEB)</td>
<td>1 μl</td>
<td>NOTE: It is essential to use a High Concentration (HC) Ligase (2 million units/ml, NEB)</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>6 μl</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>15 μl</td>
<td></td>
</tr>
</tbody>
</table>

5. Transform E.coli competent cells with 5 μl of reaction mixture, and select positive clones on kanamycin LB agar plates.

6. Identify correct clones by colony PCR and verify them by sequencing.
Sequence of one gRNA expression cassette for dicots

23-bp insert + pHEE401E

(U6-26p)-(Target-1)-(gRNA-Sc)-(U6-26t)

CGACTTGGCTTCGCCCAATACATCTCTTATAGCTTTTTTTCTCTCTCTTCGTTCATACAGTTTTTTTTTGT
TTATCAGCTTACATTTCTTGAACCCTAGCTTTCTCTTTTTAACTTTCCATTCGGAGTTTTTGTATCTT
GTTTCATATGGTGTCCCAGGATTAGATTAGGCACTGAAACCTCAAGAATTTGATTGAATAAAACATCTT
CATTCAAAGATAGATAATCTTAAAAAAAGCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTAT
ATGGGAAAGAACAATAGATTTCTTATAGCCATCGTTTTACTTTTTTTCAAACTTCAAAAGTCCCACATGC
TTAGATAAGAAAAACGAAGCTGTTATATATACAGCTAGTTTTTTTTATACAGCTAGTTTTTTTTTTTTTTTTTG
NNNNNNGTTCAGCTAGAAATAGCAGTAAAAATAAGGCTAGTCCGTTATCAACTTTGAAGAAAGTGGCACC
GAAGTCGGGTGTTTTTGCGAAAATTTTTCAGATCAGGTTCTTTTTCTCTCCTGTGTTTGGGGTTCTTTTTTTTTTTTTGT
GTTTTCTCTTCGTTTTCTGTAACTGAAACCTAAAATTTGACCTAAAAAAAATCTCAAATAATATGATTCAGTG
GTTTTGTACTTTTCAGTTAGTTGAGTTTTGCAGTTCCGATGAGATAAACCAATA

Notes:
1. Underlined letters come from binary vectors, while the others come from PCR fragments.
2. Red letters indicate primer sites.
3. Primer sequences are as follows:

**Colony PCR primers (5’→3’):**
- U6-26p-F: TGTCGCCAGGATTAGATTAGGCA
- U6-26t-R: CCCCGAAGATGACGGCGCAAGAAC
(U6-26p-F + U6-26t-R = 423 bp)

**Sequencing primers (5’→3’):**
- U6-26p-F: TGTCGCCAGGATTAGATTAGGCA