CRISPR-Cas9 Arabidopsis

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# Pipeline

# Primer design

1. Design primers for Golden Gate Assembly cloning (see <https://github.com/johanzi/crispr_cas9_arabidopsis>). 4 primers should be designed so that 2 target sites are added to the pCBC-DT1T2 vector

# Include target sites to pCBC-DT1T2

1. GoldenGate PCR with designed primers from previous step (they should be 4 primers for each reaction). Dilute the plasmid pCBC-DT1T2 to 1 ng/ul and use 1ul of this dilution for the PCR

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| **Components** | **[Initial]** | **[Final]** | **Volume (ul)** |
| dNTPs | 10mM | 200uM | 1 |
| Buffer 5X HF | 5x | 1x | 10 |
| Phusion Polymerase | 2U/ul | 1U | 0.5 |
| DT1-F0 | 1µM | 20nM | 1 |
| DT1-BsF | 20µM | 1µM | 1 |
| DT2-R0 | 1µM | 20nM | 1 |
| DT2-BsR | 20µM | 1µM | 1 |
| pCBC-DT1T2 | 1ng/ul | 1:5000 | 1 |
| H2O |  |  | 33.5 |
| Total |  |  | 50 |

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| --- | --- | --- | --- |
| **Step** | **Temperature** | **Time** |  |
| Preheating | 98°C |  |  |
| Denaturation | 98°C | 30 sec |  |
| Denaturation | 98°C | 5 sec | 30x |
| Annealing | 58°C | 10 sec |
| Amplification | 72°C | 15 sec |
| Final extension | 72°C | 5 min |  |
| Hold | 8°C |  |  |

1. Load PCR reaction onto a gel (2% agarose) and isolate the band which should be 626 bp long. Gel purify and elute in 20 ul of elution buffer. Assess concentration with a Nanodrop. The concentration of the PCR product should be about 50 ng/µl

# GoldenGate assembly of pCBC-DT1T2 and pHEE401E

1. The GoldenGate reaction allows to incorporate the PCR product of step 3 into the pHEE401E plasmid. The linearized vector backbone (pCBC-DT1T2) and the pHEE401E vector should be in 2:1 equimolar amount. However, Golden Gate Assembly process can handle 1:1 ratios (source: [NEB website](https://www.neb.com/-/media/catalog/Datacards%20or%20Manuals/manualE1600.pdf)). Considering the size of the pHEE401E vector (17 kb) and the size of the insert (626 bp), use 10x more vector than insert to get a 2:1 ratio (NEB calculator tool [here](http://nebiocalculator.neb.com/#!/ligation)).

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| **Components** | **[Initial]** | **Volume (ul)** |
| Purified PCR product | ~10 ng/µL | 2 |
| pHEE401E | ~100 ng/µL | 2 |
| T4 DNA ligase Buffer | 10X | 1.5 |
| CutSmart® Buffer | 10X | 1.5 |
| BsaI-HF v2 Enzyme | 20 U/ul | 1 |
| T4 DNA ligase | 2M U/ml | 1 |
| H2O |  | 6 |
|  | Total | 15 |

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| **Temperature** | **Time** |
| 37°C | 1 h |
| 55°C | 5 min |
| 80°C | 10 min |

For an overnight reaction (7.5 h)

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| **Temperature** | **Time** |  |
| 37°C | 5 min | 30x |
| 16°C | 10 min |
| 55°C | 5 min |  |

NB: 55°C step allows *BsaI* to perform optimally. Digesting any plasmid still present in the assembly reactions reduces background (source: NEB Golden Gate Assembly manual).

# Transformation into *E. coli*

1. Transform the 2 ul of the assembly reaction into DH5 alpha *Escherichi coli.* Plate on LB medium containing kanamycin (50 ug/ml). Check protocol <https://www.protocols.io/view/high-efficiency-transformation-protocol-c2987h-isxcefn>. Incubate plates at 37°C overnight
2. Screen 8 colonies colony by PCR reaction for genotyping. Use 1 sterile toothpick which touched one colony for the PCR and another for the liquid culture. Inoculate 4 ml of LB + kanamycin (50 ug/ml) and incubate at 37°C with shaking overnight

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| --- | --- | --- |
| **Name** | **Sequence** | **Tm** |
| T045\_pHEE\_Seq\_F | GTCACGACGTTGTAAAACGACG | 59°C |
| T046\_pHEE\_Seq\_R | CAATGATAAACCAAACGCAAATGC | 55°C |

|  |  |  |  |
| --- | --- | --- | --- |
| **Components** | **[Initial]** | **[Final]** | **Volume (ul)** |
| dNTPs | 10mM | 200µM | 1 |
| Buffer 10X BD (green) | 5X | 1X | 5 |
| my-Budget Taq pol | 5u/µL | - | 1 |
| MgCl2 | 25mM | 5mM | 10 |
| Forward Primer (T045\_pHEE\_Seq\_F) | 1µM | 20nM | 2.5 |
| Reverse Primer (T046\_pHEE\_Seq\_R) | 1um | 20nM | 2.5 |
| H2O |  |  | 28 |
|  |  | Total | 50 |

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| **Step** | **Temperature** | **Time** |  |
| Preheating | 94°C |  |  |
| Cell destruction | 94°C | 10 min |  |
| Denaturation | 94°C | 15 sec | x30 |
| Annealing | 50°C | 15 sec |
| Amplification | 72°C | 2 min |
| Final extension | 72°C | 5 min |  |
| Hold | 8°C |  |  |

The empty pHEE401E should generate an amplicon of 1999 bp. After GoldenGate Assembly, the spectinomycin resistance cassette should be replaced by the fragment of pCBC-DT1T2 and generate an amplicon of 1402 bp. Therefore, proper constructs should generate amplicons of 1402 bp.

1. Purify plasmids for 4 selected inoculated colonies with the NucleoSpin plasmid kit following manufacturer's protocol. Use 3 ml for the plasmid purification and keep 500 ul to generate a glycerol stock once the sequence is verified.
2. To verify whether no amplification error occurred during the cloning, send for Sanger sequencing the plasmids of the 4 chosen colonies to be used for Agrobacterium transformation in order to verify the sequence. Since the fragment is 1371 bp, sequence by the two ends using T045\_pHEE\_Seq\_F and T046\_pHEE\_Seq\_R primers. Verify whether the sequence of the protospacers is correct. Choose one colony with the correct sequence and prepare a glycerol stock for this colony using the culture stored at 4°C (see <https://www.addgene.org/protocols/create-glycerol-stock/>) and store at -80°C. Use the plasmid of the chosen colony to perform Agrobacterium transformation.

NB: If pCBC-DT1T2 plasmid needs to be bulked, use a ccdB resistant bacterial strain such as DB3.1 for transformation. The antibiotic used should be chloramphenicol (25 ug/ml). pHEE401E can be bulked in DH5alha using kanamycin (50 ug/ml) as selective antibiotic.

# Transformation into *A. tumefaciens*

1. Use strain pSOUP/GV3101 and check protocol (in progress). The plates for culture should contain LB + 50 ug/ml rifampicin (GV3101) + 50 ug/m gentamycin (GV3101) + 5 ug/ml tetracyclin (pSOUP) + 50 ug/ml kanamycin (pHEE401E)
2. T1 selection is done on GM full strength with hygromycin B (15µg/mL)
3. To verify the presence of the transgene in later generation (T2, T3). Use these primers

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| --- | --- |
| T128-Hygro\_G1\_F | GCGAAGAATCTCGTGCTTTC |
| T129-Hygro\_G1\_R | TCGCTAAACTCCCCAATGTC |

They generate a PCR product of 163 bp.

# Reference material

BsaI-HF v2 cat. no. R3733S, NEB