CRISPR-Cas9 Arabidopsis

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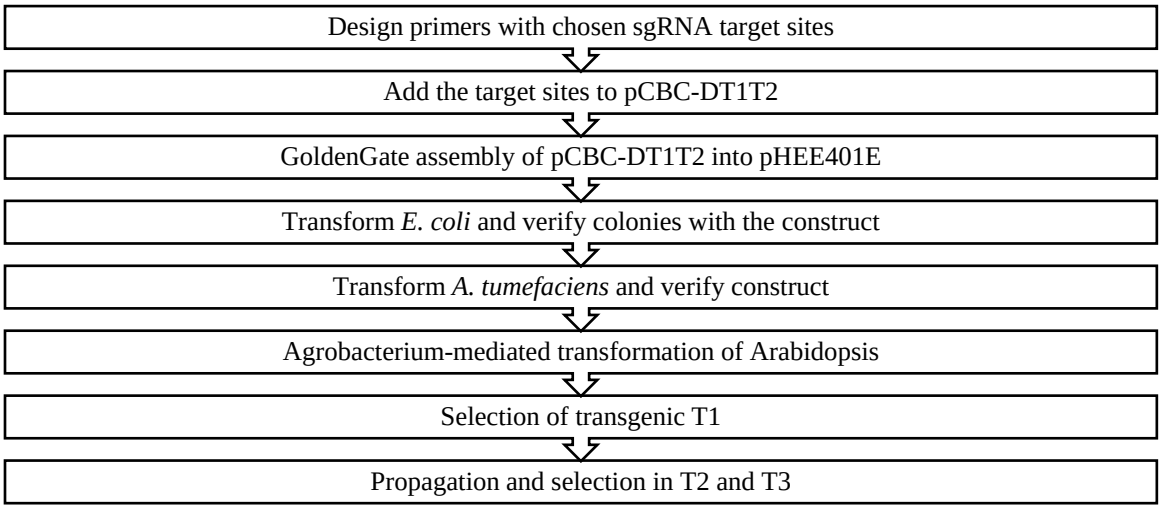
Pipeline



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

2. 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

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

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		

3. 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

4. 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). 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).

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

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

For an overnight reaction (7.5 h)

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*

5. 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

6. 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

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

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.

7. 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.

8. 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*

9. 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)

10. T1 selection is done on GM full strength with hygromycin B (15µg/mL)

11. To verify the presence of the transgene in later generation (T2, T3). Use these primers

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