CLONING GUIDELINE FOR EMPLOYING THE DICOT PE TOOL

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1. List of materials required (see also annex 1)

- **Level 0:** pICH41331::pU6cm-pegR::EURb7 (https://www.addgene.org/227695/)
- **Level 1:** pICSL11024 (pNOS-NptII-tOCS) (https://www.addgene.org/51144/); pICH47742::p35S-PE2max-NC::tEURb7 (https://www.addgene.org/227693/);
- **Level 1, position 3 acceptor plasmid:** pICH47751 (https://www.addgene.org/48002/).
- **End-linker 3**: pICH41766 (https://www.addgene.org/48018/).
- **Binary acceptor vector**: pLSL.R.Ly geminiviral replicon (https://www.addgene.org/227697/) or conventional T-DNA pAGM4723 (https://www.addgene.org/48015/).
- Q5® High-Fidelity DNA Polymerase (NEB).
- NEB T4 DNA Ligase (M0202).
- NEB BsaI-HF®v2 (R3733).
- ThermoScientific BpiI (ER1012).
- dNTP Mix (10 mM each).
- MQ or double-distilled water.
- PCR machine.
- E. coli competent cells.
- Spectinomycin.
- Carbenicillin or Ampicillin.
- Kanamycin.
- X-gal (10mg/ml).
- LB liquid and LB agar.
- PCR product cleanup kit and plasmid isolation mini kit

2. Cloning gRNA and RTT into the pU6cm acceptor vector (see annex 2 before start)

- Acceptor vector : pICH41331::pU6cm-pegR::EURb7 (Level 0).
- gRNA and RTT: prepared by PCR, not oligo annealing.

Design for synthesizing oligos containing complementary portions, such as:

-For gRNA:

gR-Fwd: CAGTC**GAAGAC**AA**TGCA**xxxxxxxxxxxxxxxxxxxx

gR-Rev: CAGTC**GAAGAC**AAAAACyyyyyyyyyyyyyyyyyyyy

xxxxxxxxxxxxxxxxxxxx: spacer sequence; y: reverse complement of x.

-For RTT:

RTT-Fwd: CAGTCGAAGACAAGTGCnnn…

RTT-Rev: CAGTCGAAGACAACGCGmmm…

nnn…: 5' part of the RTT sequence

mmm…: reverse complement 3' part of the RTT sequence.

Nnn… and mmm… are designed to contain overlapped, complementary sequences with Tm=55-60 °C.

Preparation of gRNA and RTT by PCR:

-Order the oligos and dilute them to a 10µM working solution.

-Prepare the PCR reaction as follows:

Component	**1x**
5x Q5 buffer	3
dNTPs (10mM)	0.3
F-P	1
R-P	1
Template	0
H2O	9.55
Q5® High-Fidelity DNA Polymerase	0.15
Total	15

-Thermocycling:

Step	**Temp. (°C)**	**Time**	**Cycle number**
1	98	30''	1
2	98	8''	25
	Tm of oligos	20''	
	72	8''	
3	72	1'	1
4	12	30'	1

-Purify the PCR products:

- Using a direct PCR cleanup kit. Elute with 60-80 µl elution buffer.
- Measure concentrations using a Nanodrop and adjust concentrations to ~ 3-5 ng/µl.

Assemble the gRNA and RTT sequences into the pU6cm acceptor vector:

-Prepare Golden Gate reaction mixture according to the following table:

Comp	Size (bp)	fmol for 100ng	Conc.	Required amount (fmol)	Required amount (ng)	1x
10x T4 DNA buffer			10x			1.50
gR PCR product	54	2814.8	3.0	20	0.71	0.24
RTT PCR product	60*	2533.3	5.0	20	0.79	0.16
pICH41331::pU6cm-pegR::EURb7	4534	33.5	217.0	13	38.78	0.18
T4 DNA ligase						0.50
BpiI						0.50
H2O						11.93
Sum						15.00

*Adjust this length according to your RTT.

-Thermocycling:

Step	Temperature (oC)	Time (minute)	Cycle	Note
1	37	15	1	Pre-digestion
2	37	2	30	Digestion-ligation
	16	5		
3	37	5	1	Post-digestion
4	50	5	1	Post-digestion
5	80	5	1	Denaturation

-Transform all the reaction mixture into ~60ul 10beta or DH5alpha E. coli competent cells using a heat shock protocol (Add the reaction mixture into the competent cell tube, mix well and incubate on ice for 30 minutes; Heat shock in 42oC water bath for 1 minute and imediately transfer the tube on ice and incubate for 3 minutes; Add 600-800 ul LB to the tube and incubate on a shaker (180 rpm) at 37oC for 30 minutes). Spread ~100-150 µL of the culture onto an LB agar plate containing 75 mg/L spectinomycin.

-Pick three colonies and incubate in 2.1 ul LB + 75 mg/L spectinomycin at 37oC, 180rpm shaking overnight.

-Isolate plasmids from the cells.

-Digestion checking with NheI&PmlI (expected bands: ~3800- and ~800-bp) or appropriate RE pairs. This step is optional.

-Confirm by sequencing with appropriate primer, such as pCmYLCV-sF1 (5'-GTCAAGGCGGCGAAGTATT-3').

3. Cloning the aepegRNA expression cassette into the level 1 vector

Set up a Golden Gate reaction as follows:

Comp	Size (bp)	fmol for 100ng	Conc.	Required amount (fmol)	Required amount (ng)	1x
10x T4 DNA buffer			10x			1.50
pICH41331_pU6cm-aepegRNA-EURb7 (confirmed clone)	4520*	33.6	150.0	20	59.47	0.40
pICH47751**	4968	30.6	150.0	13	42.49	0.28
T4 DNA ligase						0.50
BsaI						0.50
H2O						11.82
Sum						15.00

*Adjust this length according to your pegRNA. **pICH47751 is a level 1 position 3 vector (addgene.org/48002/) of the Moclo kit (https://www.addgene.org/browse/article/7094/). If one wants to clone the pegRNA into another position, the corresponding plasmids can be found in the kit.

-Thermocycling:

Step	Temperature (oC)	Time (minute)	Cycle	Note
1	37	20	1	Pre-digestion
2	37	2	30	Digestion-ligation
	16	5		
3	37	5	1	Post-digestion
4	50	5	1	Post-digestion
5	80	5	1	Denaturation

-Transform all the reaction mixture into ~60ul 10beta or DH5alpha E. coli competent cells using a heat shock protocol (Add the reaction mixture into the competent cell tube, mix well and incubate on ice for 30 minutes; Heat shock in 42oC water bath for 1 minute and imediately transfer the tube on ice and incubate for 3 minutes; Add 600-800 ul LB to the tube and incubate on a shaker (180 rpm) at 37oC for 30 minutes). Spread ~100-150 µl of the culture with 25µl X-gal (10mg/ml) onto an LB agar plate containing 100 mg/L carbenicillin or ampicillin.

-Pick three white colonies (negative colonies are blue) and incubate in 2.1 µL LB + 100 mg/L carbenicillin or ampicillin at 37 °C, 180rpm shaking overnight.

-Isolate plasmids from the cells.

-Digestion checking with BpiI (expected bands: 4352- and ~2300-bp).

4. Assembly of the level 1 expression cassette into the pLSL.R.Ly binary vector for plant transformation

Set up a Golden Gate reaction as follows:

Comp	Size (bp)	fmol for 100ng	Conc.	Required amount (fmol)	Required amount (ng)	1x
10x T4 DNA buffer			10x			1.50
pICSL11024 (pNOS-NptII-tOCS)	6243	24.3	200.0	20	82.14	0.41
pICH47742::p35S-PE2max-NC::tEURb7	13523	11.3	400.0	20	177.63	0.44
pICH47751_pU6cm-aepegRNA-Eurb7(confirmed clone)	6686	22.7	200.0	20	87.97	0.44
pICH41766	3318	45.8	150.00	20	43.66	0.29
pLSL.R.Ly*	15031	10.1	400.0	13	128.55	0.32
T4 DNA ligase						0.50
BpiI						0.50
H2O						10.75
Sum						15.00

*pLSL.R.Ly is a geminiviral replicon acceptor vector. pAGM4723 (addgene.org/48015/) is often used for a conventional T-DNA acceptor plasmid.

-Thermocycling:

Step	Temperature (oC)	Time (minute)	Cycle	Note
1	37	20	1	Pre-digestion
2	37	2	32	Digestion-ligation
	16	5		
3	37	5	1	Post-digestion
4	50	5	1	Post-digestion
5	80	5	1	Denaturation

-Transform all the reaction mixture into ~60ul 10beta or DH5alpha E. coli competent cells using a heat shock protocol (Add the reaction mixture into the competent cell tube, mix well and incubate on ice for 30 minutes; Heat shock in 42oC water bath for 1 minute and imediately transfer the tube on ice and incubate for 3 minutes; Add 600-800 ul LB to the tube and incubate on a shaker (180 rpm) at 37oC for 30 minutes). A centrifugation was used to collect the cells and spread them all onto an LB agar plate containing 75 mg/L kanamycin.

-Pick three white colonies (negative colonies are red/pink) and incubate in 2.1 μL LB + 75 mg/L kanamycin at 37 °C, 180rpm shaking overnight. Note: if the pLSL.R.Ly acceptor is used, the correct colonies often grow slower and thus smaller and thinner than others.

-Isolate plasmids from the cells.

-Digestion checking with XhoI&SacII (expected bands are ~10160-, 7038, and 3113-bp) or appropriate REs.

Annex 1

Plasmid vectors deposited to Addgene

No.	Name of vector	Description	Position	Addgene number	Antibiotic for bacterial culture
1	pICH47742::p35S-ePEmax3::tEURb7	Level 1 position 2: ePEmax3 being driven by long CaMV 35S (p35S) and terminated by a dual terminator (EURb7)	Position 2, PE protein variant	227692	Ampicillin/Carbenicillin
2	pICH47742::p35S-PE2max-NC::tEURb7	Level 1 position 2: PE2max-NC being driven by long CaMV 35S (p35S) and terminated by a dual terminator (EURb7)	Position 2, PE protein variant	227693	Ampicillin/Carbenicillin
3	pICH47742:: pRPS5a-PE2max-NC::tHSP	Level 1 position 2: PE2max-NC being driven by pRPS5a and terminated by tHSP	Position 2, PE protein variant	227694	Ampicillin/Carbenicillin
4	pICH41331::pU6cm-pegR::EURb7	Level 0 acceptor vector for cloning altered epegRNA: altered epegRNA being driven by U6 composite promoter (pU6cm) and terminated by a dual terminator (EURb7)	Altered epegRNA expression cassette, cloning vector	227695	Spectinomycin
5	pLSL.R.Ly	Reengineered geminiviral replicon vector	Binary vector	227697	Kanamycin

Annex 2

IMPORTANT NOTES

1. Using the Golden Gate cloning method to assemble the expression cassettes.
2. In all Golden Gate reactions, the amount of each insert is 20 fmol regardless of the number of inserts, and the acceptor vector amount is 13 fmol.
3. These are two level 1, position 2 golden gate plasmids for the PE protein expression cassettes, a level 0 acceptor vector for cloning gRNA and RTT into the altered epegRNA expression cassette, and a level 2 replicon acceptor vector (pLSL.R.Ly).
4. We recommend testing ePEmax3 and PE2max-NC configurations in the initial experiment. It is important to note that PE2max-NC may lead to a higher PE rate at the transformant screening stage, albeit it may also result in a greater number of events containing by-products.
5. We designed the PBS and RTT based on the most updated information in the field and suggested the criteria in our recent PE review (https://onlinelibrary.wiley.com/doi/full/10.1111/pbi.14188).
6. The altered epegRNA expression cassette contains two BpiI cloning sites for (1) spacer and (2) RTT. To clone the altered epegRNAs, we chemically synthesized the pU6cm::aepegRNA fragment with dual cloning sites (BpiI) for spacer and RTT sequences. Then, we inserted it into the level 0 Moclo acceptor vector pICH41331 with the EuRB7 terminator, resulting in the pICH41331::pU6cm-pegR::EuRB7 acceptor vector.