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 We use this protocol and it's working

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Loop L2 (even level) SapI type IIS cloning into pCs vectors

Forked from [Loop L2 \(even level\) type IIS cloning - pCs-ye vectors](#)

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ABSTRACT

Protocol based on

[Pollak B, Cerda A, Delmans M, et al \(2019\) Loop assembly: a simple and open system for recursive fabrication of DNA circuits. New Phytol 222:628–640](#)

<https://doi.org/10.1111/nph.15625>

MATERIALS

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☒ Sterile water **Contributed by users**

☒ dATP, 100mM, 25uMoles **Promega Catalog #U1205**

☒ BSA, molecular biology grade, 20 mg/ml **New England Biolabs Catalog # B9000S**

☒ Tango Buffer **Thermo Fisher Scientific Catalog #BY5**

☒ T4 DNA ligase **Thermo Fisher Scientific Catalog #15224041**

☒ LguI (SapI) **Thermo Fisher Scientific Catalog #ER1931**

Loop pCs vectors

Loop vectors for nuclear transformation: pCs (A) and for chloroplast transformation pCsChlo (B). Loop fusion sites in the pCs vectors to assemble different L1 constructs into a L2 construct using a pCs vector and *SapI* are: a (ATG) and o (GCT).

Loop fusion sites in the pCs vectors to assemble different L2 constructs into a L3 construct using a pCs vector and *BsaI* are: A (GGAG), B (TACT), C (AATG), E (GCTT) and F (CGCT).

Left (LB) and right border (RB) repeats from nopaline C58 T-DNA for *Agrobacterium*-mediated nuclear transformation. SmR: spectinomycin bacterial resistance cassette. *LacZ*: *lacZ* α cassette for blue-white screening of colonies.

Example of assembly of L1 constructs into a L2 device

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Loop assembly of four transcription units (L1) into a L2 device using a pCs plasmid and Sapl.

Protocol for assembly of L1 constructs into a L2 device

- 3 Determine the concentration of each DNA plasmid needed (L1 plasmids and pCs acceptor plasmid) with spectrophotometry (Nanodrop).
In the example in step 2, determine concentration of plasmids L1_001-Ck1, L1_002-Ck2, L1_003-Ck3, L1_004-Ck4 and pCsA
- 4 Prepare aliquots for each plasmid at a concentration of 15 nM for the L1 plasmids and of 7.5 nM for the acceptor pCs vector. With this final concentration, 1 μ L of each plasmid is added to the

plasmids mix (see step 6).

To calculate the concentration in ng/μL:

- For a final concentration of 15 nM, the concentration in [ng/ul] equals N (the length in bp of the plasmid) divided by 110. This is an approximation of the formula:

$$15 \cdot 10^{(-9)} \text{mol/L} \times ((607.4 \times N) + 157.9) \text{g/mol} \times 10^{(-6)} \text{L/}\mu\text{L} \times 10^9 \text{ng/g} = \text{concentration (ng/}\mu\text{L)}$$

- For a final concentration of 7.5 nM, the concentration in [ng/ul] equals N divided by 220.

5 Prepare the Loop assembly Even Level reaction master mix (MM) according to according to Table

Component	Volume (μL)
Sterile water	2
10x Tango buffer (Thermo Fisher)	1
1 mg/mL bovine serum albumin (NEB)	0.5
T4 DNA ligase (5 U/μL) (Thermo Fisher)	0.25
10mM ATP (SIGMA)	1
SapI (LguI) (5 U/μL) (Thermo Fisher)	0.25
Final volume	5

6 Prepare plasmids mix by adding in a 0.2 mL tube: 1 μL of each of the 4 L1 plasmids , and 1 μL of the pCs (see step 2). Mix well.

7 Add 5 μL of master mix (step 5) to the 5 μL of plasmids mix (step 6), to a final volume of 10 μL. Mix well.

- 8** Place samples in a thermocycler and use the following program:
Assembly: 26 cycles of 37 °C for 3 min and 16 °C for 4 min.
Termination and enzyme denaturation: 50 °C for 5 min and 80 °C for 10 min.
- 9** Transform 20 µL of chemically competent *E. coli* cells (transformation efficiency of 1×10^7 transformants/µg plasmid DNA) using 2 µL of the Loop assembly reaction and then plate on LB agar plates containing 100 µg/mL spectinomycin and 40 µg/mL of X-gal for blue-white screening.
- 10** Incubate O/N at 37 °C.
- 11** Colonies with white color are likely to contain a L2 insert cloned into the pCs vector (In the example in step 2: 001-002-003-004)
Blue color colonies will contain undigested pCs vector with LacZ
- 12** Confirm the presence of the correct insert with Sanger sequencing using the primers pC_F (GCAACGCTCTGTCATCGTTAC) and pC_R (GTAACCTTAGGACTTGTGCGACATGTC) for pCs vectors, and pC_F and pC_R2 (CAATCTGCTCTGATGCCGCATAGTTAAG) for pCschlo vectors.