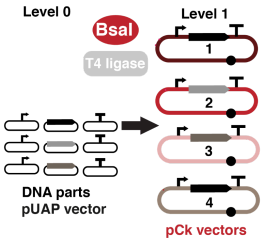
Dec 03, 2019 Version 2

Loop L1 (odd level) BsaI type IIS cloning into pCk vectors V.2



Version 1 is forked from Loop L1 (odd level) type IIS cloning - pCk-ye vectors

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Eftychis Frangedakis[1], Susana Sauret-Gueto[2], Anthony West[3], Nicola Patron[4], Marta Marta Tomaselli[2], Marius Rebmann[2], Jim Haseloff[2]

[1]University of Cambridge; [2]Plant Sciences, University of Cambridge, OpenPlant; [3]previously at Earlham Institute, Norwich; [4]Earlham Institute, Norwich

OpenPlant Project

Susana Sauret-Gueto
Plant Sciences, University of Cambridge, OpenPlant

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Protocol status: Working
We use this protocol and it's working

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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

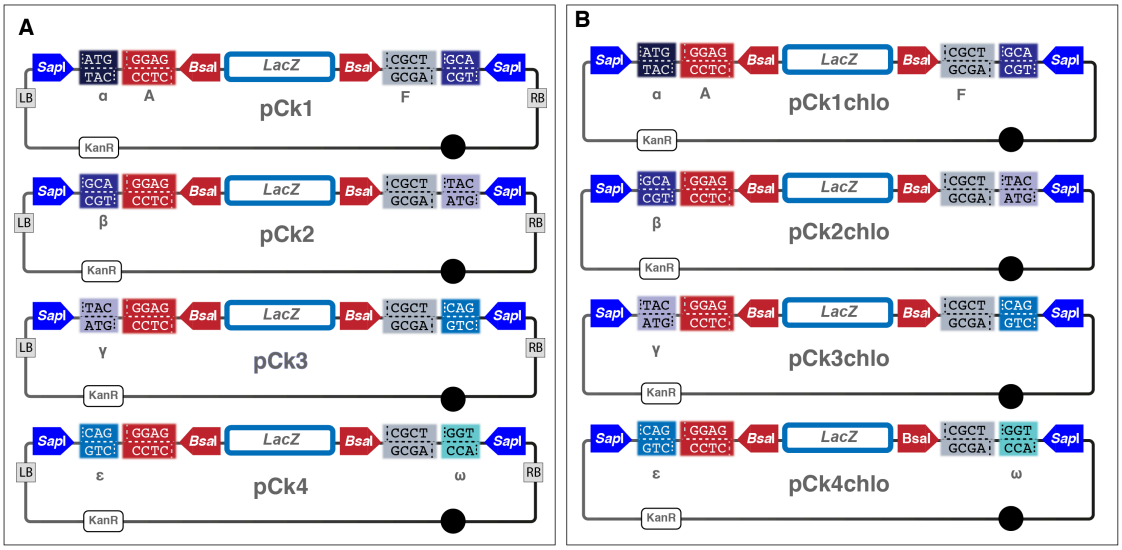
Materials

MATERIALS

- BsaI - 5,000 units **New England Biolabs Catalog #**R0535L
- T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S
- Sterile water
- BSA, molecular biology grade, 20 mg/ml **New England Biolabs Catalog #** B9000S
- 10X NEB T4 DNA ligase buffer **New England Biolabs**

Loop pCk vectors

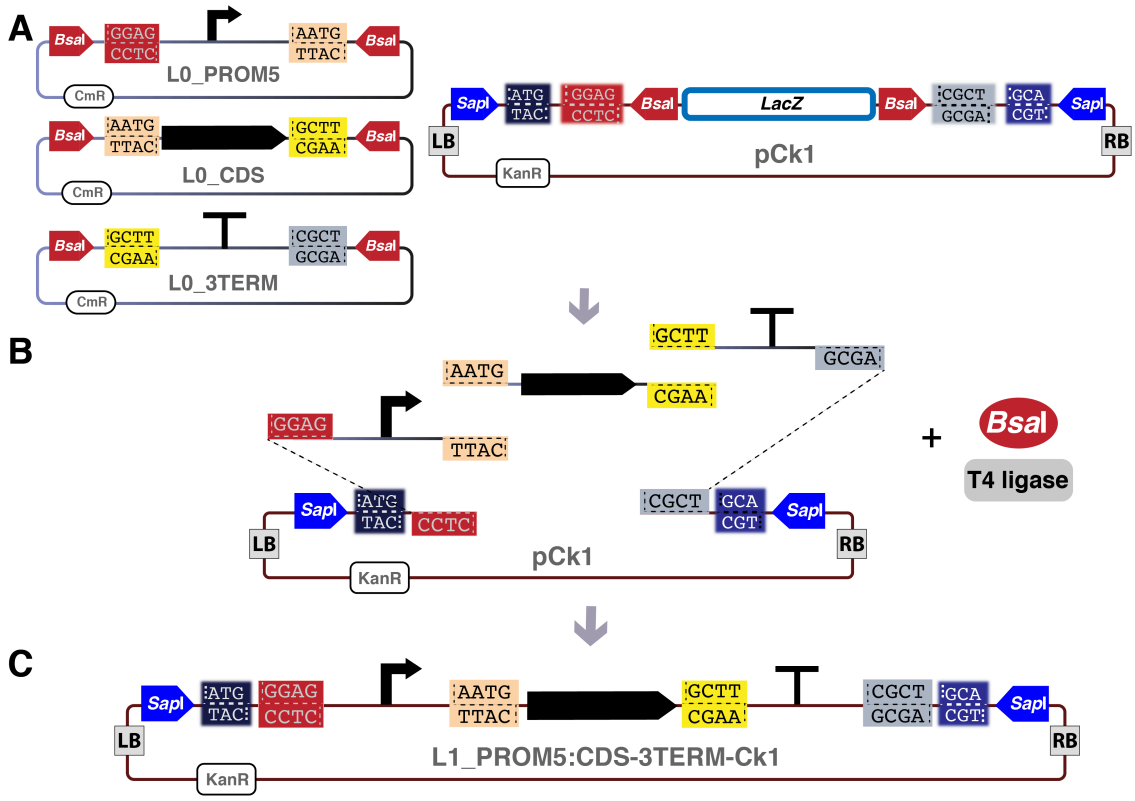
1



Loop vectors for nuclear transformation: pCks (A) and for chloroplast transformation pCkchlo (B).
Loop fusion sites in the pCk vectors to assemble different L0 parts into a L1 construct using a pCk vector and BsaI are: A (GGAG) and F (CGCT).
Loop fusion sites in the pCk vectors to assemble different L1 constructs into a L2 construct using a pCs vector and SapI are: a (ATC), b (GCA), d (TAC), e (CAG) and o (GCT).
Left (LB) and right border (RB) repeats from nopaline C58 T-DNA for Agrobacterium-mediated nuclear transformation. KanR: kanamycin bacterial resistance cassette. LacZ: lacZα cassette for blue-white screening of colonies.

Example of assembly of L0 parts into a transcription unit (L1)

2



Loop assembly of multiple L0 parts into a transcription unit (L1) using a pCk plasmid and BsaI.

Protocol for assembly of L0 parts into a transcription unit (L1)

3 Determine the concentrations of each DNA plasmid needed (L0 plasmids and pCk acceptor plasmid) by spectrophotometry (Nanodrop).
In the example in step 2, determine concentration of plasmids L0_PROM5, L0_CDS, L0_3TERM and pCk1.

4 Prepare aliquots for each plasmid at a concentration of 15 nM for the L0 plasmids and of 7.5 nM for the acceptor pCk vector. With this final concentration, 1 µL 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:
15·10^(-9)mol/L x ((607.4 x N) + 157.9)g/mol x 10^(-6)L/µL x 10^9ng/g = concentration (ng/µL)

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

5 Prepare Loop assembly Level 1 reaction master mix (MM) according to Table , if four or less number of L0 parts are assembled into a pCk vector (otherwise see step 8)

	Components	Volume (µL)
	Sterile water	3
	10x T4 ligase buffer (NEB)	1
	1 mg/mL bovine serum albumin (NEB)	0.5
	T4 DNA ligase at 400 U/µL (NEB)	0.25
	10 U/µL BsaI (NEB)	0.25
	Final volume	5

6 Prepare plasmids mix by adding in a 0.2 mL tube: 1 µL of each L0 plasmid , 1 µL of the pCk vector (see step 4), and sterile water up to 5 µL. Mix well.

When 4 L0 parts are assembled into a pCk plasmid, the volume of the plasmid mix is 5 µL, and thus no volume of water is added.

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

8 If more than 4 L0 parts are to be assembled into a pCk vector, reduce the water volume in the MM by 1 µL (step 5) for each extra 1 µL of DNA part added in the plasmids mix (step 6).

9 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.

10 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 50 µg/mL kanamycin and 40 µg/mL of X-gal for blue-white screening.

11 Incubate overnight at 37 °C.

12 Colonies with white color are likely to contain an L1 insert cloned into the pCk vector (In the example in step 2: PROM5:CDS-3TERM)
Blue color colonies will contain undigested pCk vector with LacZ

13 Confirm the presence of the correct insert with Sanger sequencing using the primers pC_F (GCAACGCTCTGTCATCGTTAC) and pC_R (GTAACTTAGGACTTGTGCGACATGTC) for pCk vectors, and pC_F and pC_R2 (CAATCTGCTCTGATGCCGCATAGTTAAG) for pCkchlo vectors.