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Loop and uLoop assembly V.4

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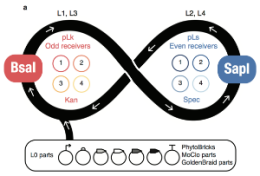

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We use this protocol and it's working

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Abstract

This protocol is used for performing Type IIS assembly by either *BsaI* or *SapI*-mediated restriction/ligation using Loop assembly with either Loop or uLoop plasmids.

Loop assembly comprises 8 receiver plasmids in odd and even levels (4 per level), which contain directional overhangs for higher level assembly. Odd and even level receiver plasmids are used in a recursive schema for assembly in iterative 'loops'. Any assembly (except for L0 part composition into a L1 transcriptional unit) requires the usage of 4 donor plasmids (each in a specific position) and one receiver plasmid. *BsaI* is used for assembly into odd levels using kanamycin selection, and *SapI* is used in even levels using spectynomycin selection. Loop odd receivers use the Common Syntax standard for L0 part assembly into TUs (Patron, *et al.* 2015 [10.1111/nph.13532]). Then, 4 L1 plasmids (positions 1-4) are assembled into an even receiver plasmid to provide a L2 assembly (4 TUs). For higher level assemblies the same assembly structure is followed, 4 L2 plasmids (positions 1-4) with an odd receiver to yield a L3 assembly (16 TUs).

The Loop type IIS assembly protocol is based on:
“Patron, NJ (2016) DNA Assembly for Plant Biology. Current Protocols in Plant Biology 1:1-13 [doi: 10.1002/cppb.20038]”, but with slight modifications for DNA concentrations:

This protocol uses a target amount of each donor DNA of 15 fmol and 7.5 fmol for receiver plasmid DNA in a 10 µL reaction. In order to perform accurate dispensing, the plasmid DNA is diluted to their corresponding concentration of 15 fmol/µL for donor parts and of 7.5 fmol/µL for the receiver plasmid, and then 1 µL of each plasmid is added to the DNA mix. The plasmid part target concentration (in ng/uL) equals to each donor plasmid length / 100, and of the receiver plasmid length / 200.

Volumes CAN be reduced by half, but expect more variability from aliquoting.

Guidelines

Each level (odd and even) contains four 'receiver' plasmids:

pOdd-1, 2, 3, 4
pEven-1, 2, 3, 4

For a the assembly of L0 parts into a L1 TU, a variable number of parts can be used for the asssembly of the TU, however, for higher level assemblies only 4 donor modules can be assembled into any receiver.

Use only high-purity plasmid preparations. Genomic contamination of plasmid DNA will interfere with assembly reactions.

Materials

MATERIALS

- T4 DNA Ligase Reaction Buffer - 6.0 ml **New England Biolabs Catalog #**B0202S
- BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #**B9000S
- BsaI - 1,000 units **New England Biolabs Catalog #**R0535S
- SapI - 250 units **New England Biolabs Catalog #**R0569S
- Ultrapure Distilled, Nuclease Free Water
- X-Gal **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B4252
- T4 DNA Ligase **New England Biolabs Catalog #**M0202

Safety warnings

Avoid several freeze/thaw cycles and replace 10X T4 ligase buffer every month or so. Replace enzymes every 1 or 2 months. Protect X-Gal from light. DMF and DMSO are toxic, be careful when handling X-Gal.

Before start

Do not use BsaI-HF, that enzyme will NOT work with this reaction. Use either BsaI or BsaI-HFv2.

Thaw 10X T4 ligase buffer at RT and then leave on ice. Keep BSA on ice. Briefly vortex 10X ligase buffer before using. Use aliquoted volumes of T4 ligase Buffer since ATP and DTT will degrade over short amounts of time (less than a month) affecting the efficiency of the reaction.

Calculate plasmid target concentrations

1 The required concentration for each donor part in ng/µL is plasmid length / 100. That will yield a concentration of 15 fmol/µL. For the receiver plasmid the target concentration is length / 200, yielding 7.5 fmol/µL.

Prepare DNA mixture

2 Add into a PCR tube:
1 µL of each donor part at 15 fmol/µL.
1 µL of receiver plasmid at 7.5 fmol/µL.

Add water up to 5 µL (assemblies from L1 and above do not need adjustment since 5 plasmids are used)

Master mixes

3 Odd and even level assemblies use different formulations due to specific requirements of the restriction enzymes used.

For odd levels (BsaI or BsaI-HFv2), 10x T4 DNA ligase buffer is used.
For even levels (SapI), 10x Cutsmart buffer is used mixed with 10x T4 DNA ligase buffer in a 1:1 ratio.

For BSA (bovine serum albumin), check stock concentration. NEB 'molecular biology grade BSA' comes at 20 mg/mL, depreciated NEB purified BSA comes at 10 mg/mL. Prepare 20 µl of 1 mg/mL diluted BSA in water solution.

Thaw buffers at RT, then place on ice.

IMPORTANT:

Do not use BsaI-HF, that enzyme will NOT work with this reaction. Use either BsaI or BsaI-HFv2.

Use aliquoted volumes of T4 Buffer since ATP and DTT will degrade over short amounts of time (less than a month) affecting the efficiency of the reaction.

If low efficiency is seen, use double the restriction enzyme volumes.

Prepare reaction master mix

4 of reactions to be performed and include an extra reaction to account for pipetti5n.g error3s.
For each odd level reaction:

Odd level BsaI-Ligase 2x master mix:

	Reagent	Concentration	Volume (µL)
	HPLC H_20		3
	T4 DNA ligase buffer	10X	1
	Diluted BSA (Bovine Serum Albumin)	1 mg/mL	0.5
	T4 DNA ligase	400 U/µL	0.25
	BsaI (or BsaI-HFv2)	10 U/µL	0.25

Even level SapI-Ligase 2x master mix:

	Reagent	Concentration	Volume (µL)
	HPLC H_20		3.5
	Cutsmart buffer	10X	0.5
	T4 DNA ligase buffer	10X	0.5
	T4 DNA ligase	400 u/µL	0.25
	SapI	10 U/µL	0.25

Mix by pipetting (enzymes in glycerol will be in the bottom of the tube). Then pipette from the top for accurate dispensing.

Mix DNA and reaction master mix

5 Add the 5 µL of the corresponding reaction master mix to the 5 µL of DNA mix and mix thoroughly by pipetting.

Incubate reaction

6 Place the reaction on a thermocycler (PCR machine). And run the following program:

Step 1 - 37 ºC for 3 min
Step 2 - 16 ºC for 4 min
Repeat 25x step 1 and 2
Step 3 - 50 ºC - 5 min

Step 4 - 80 ºC - 10 min

Note: number of cycles and incubation times can be adjusted but the reaction efficiency may decrease.

Transform and plate

7 Use 1 µL of reaction to transform chemically competent cells.

Recover in SOC for 1 hour incubating at 37 ºC in a shaking incubator.

Prepare plates with respective antibiotics with 50 ug/mL of X-GAL and 1 mM IPTG.

Pick colony grow overnight and validate

8 Pick 2 white colonies for each construction and grow in 10 mL of LB + respective antibiotic and perform plasmid purification. Validate assembly by means of restriction digest. Suggested enzymes: ThermoFischer XbaI and PstI FastDigest enzymes. If higher concentrations are required, increase inoculation volume and perform Midi prep as directed by manufacturer's instructions.