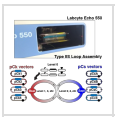

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Loop assembly using Labcyte Echo 550

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1 Works for me dx.doi.org/10.17504/protocols.io.8zvhx66

OpenPlant Project

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ABSTRACT

Loop L1 and L2 type IIS assembly protocol, automated and minituarised for assembly of 500nl final volume reactions using the Labcyte Echo 550.

MATERIALS

NAME	CATALOG #	VENDOR
BsaI - 5,000 units	R0535L	New England Biolabs
T4 DNA Ligase - 20,000 units	M0202S	New England Biolabs
Sterile water		
dATP, 100mM, 25uMoles	U1205	Promega
BSA, molecular biology grade, 20 mg/ml	B9000S	New England Biolabs
10X NEB T4 DNA ligase buffer		New England Biolabs
384-Well Low Dead Volume (LDV) Microplate	LP-0200	
Tango Buffer	BY5	Thermo Fisher Scientific
T4 DNA Ligase (5 U/µL)	15224041	Thermo Fisher
LguI (SapI) (5 U/µL)	ER1932	Thermo Fisher

MATERIALS TEXT

384 PP plate labcyte

384 well skirted PCR plate (4ti-0384)

PCR foil seal (4ti-0550)

breath-easy sealing membrane (Z30059-1PAK)

1 **Before using the Echo to do Loop assemblies, you need a list of all Loop reactions that needs doing, and all plasmids needed.** Thus, make a csv files with all Loops that need doing. Separate Loop1 from Loop2. Examples:

L1 construct	Accpetor plasmid (AP)	L0 plasmid in position 1	L0 plasmid in position 2	L0 plasmid in position 3	L0 plasmid in position 4	L0 plasmid in position 5
L1_001-Ck1	pCk1	PROM5_a	CDS_a	3TERM_a		
L1_002-Ck2	pCk2	PROM5_a	CDS12_b	CTAG_b	3TERM_a	
L1_003-Ck2	pCk2	PROM5_b	CDS12_b	CTAG_b	3TERM_a	
L1_004-Ck2	pCk2	PROM_c	5UTR_c	CDS12_b	CTAG_b	3TERM_a

Example for L1 Loop

L2 construct	Accpetor plasmid (AP)	L1 plasmid in position 1	L1 plasmid in position 2	L1 plasmid in position 3	L1 plasmid in position 4
L2_001-CsA	pCsA	L1_001-Ck1	L1_002-Ck2	L1_005-Ck3	L1_006-Ck4
L2_002-CsA	pCsA	L1_001-Ck1	L1_003-Ck2	L1_005-Ck3	L1_006-Ck4

Example for L2 Loop

2 **Next create LDV source plate that contain all plasmids needed but the APs.**

For L1 Loops, have a LDV source plate with the libary of L0 parts.
For L2 Loops, have a LDV source plate with the library of L1 plasmids.

Use Echo calibration 384LDV_AQ_B2 for these plates.

LDV plates working range is~ 3-12 ul (maximum volume is 12 ul and dead volume is 3 ul).
Thus, fill in the wells wtih 10 ul of a given plasmid.

Source plasmids final concentration is 15 nM.

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)

This plate can be sealed and kept at -20C, for reuse in the future as many times as necessary, and empty wells can be filled in with new plasmids.

Keep a csv files with the LDV plate source well and sample name and update as you fill in new positions.

3 **Next create PP source plate with the APs and MM and water.**

For L1 Loops, have a PP source plate with the pCk APs and L1-MM-BsaI.
For L2 Loops, have a PP source plate with the pCs APs and L2-MM-SapI.

Use Echo calibration 384PP_AQ_BP2 for these plates.

PP plates working range is ~ 15-65 ul (maximum volume is 65 ul and dead volume is 15 ul).
Thus, fill in the wells wtih ~30-50 ul of a given plasmid.
MM contains enzymes, BSA and glycerol, and has a higher tendency to creep up the walls of the well than water, thus the dead volume is higher, at around 18 ul (always check the dead volume for your MM). The dead volume is the volume at which the transducer of the Echo can not detect the top of the meniscus anymore and thus can not focus to transfer droplets by accoustic energy.

Prepare a source 384 PP plate with APs (pCks, pCs) at a final concentration of 7.5 nM and Loop plasmids with spacers (pCk1-spacer, pCk2-spacer,....) at a final concentration of 15 nM.
Use two wells in this plate for water and for the master mix (MM).
This plate can sealed, kept at -20C, and reused, and every time a new Loop-Echo assembly needs doing, choose a new pair of wells for water and MM. Alternatively have two different PP plates, one for the APs, and one for the water and MM.

Keep a csv files with the PP plate source well and sample name and update as you fill in new positions.

4 **Final volume of L2-Loop reactions at the Echo is 500 nl**, with 250 ul of MM (see next step on preparing MM) and 250 ul of plasmids (50 ul of each one of the 5 plasmids), as follows:

Plasmid	Volume
pCk2	50 nl
L1_001-Ck1	50 nl
L1_002-Ck2	50 nl
L1_004-Ck3	50 nl
L1_005-Ck3	50 nl
L2_MM-SapI	250 nl
Final volume	500 nl

Example of volumes dispensed by Echo for to assemble L2_001-CsA

5

For L2 Loops, prepare MM as follows:

	Volume for 1 reaction in 1 well in a 384 PCR plate	Volume for 96 reactions in a 384 PCR plate
Water	100 nl	9.6 ul
10x Tango buffer (Thermo Fisher)	50 nl	4.8 ul
1 mg/mL bovine serum albumin (NEB)	25 nl	2.4 ul
10mM ATP (SIGMA)	50 nl	4.8 ul
T4 DNA ligase (5 U/µL) (Thermo Fisher)	12.5 nl	1.2 ul
SapI (LguI) (5 U/µL) (Thermo Fisher)	12.5 nl	1.2 ul
Final volume	250 nl	24 ul

L2-MM-SapI

To prepare the MM always consider the volume you need for the plate reactions plus the dead volume of the MM solution in a PP plate.

Always prepare the MM in an eppendorf tube first, mix by pipetting and then transfer the MM to the PP-well. This is to ensure all components of the MM are well mixed.

6 **Final volume of L1-Loop reactions at the Echo is 500 nl.** In this case, because number of plasmids needed is variable (4-7) the MM volume needs to change depending on that. Thus, if we want to do different L1-Loops with different number of plasmids, we can assume a 6 plasmids for all, and use water for the positions without a plasmid as whown below.

L1-Loop with 6 plasmids		L1-Loop with 5 plasmids		L1-Loop with 4 plasmids	
pCk2	50 nl	pCk2	50 nl	pCk2	50 nl
PROM_c	50 nl	PROM5_b	50 nl	PROM5_a	50 nl
5UTR_c	50 nl	CDS12_b	50 nl	CDS_a	50 nl
CDS12_b	50 nl	CTAG_b	50 nl	3TERM_a	50 nl
CTAG_b	50 nl	3TERM_a	50 nl	water	100 nl
3TERM_a	50 nl	water	50 nl		
L1_MM6-BsaI	200 nl	L1_MM6-BsaI	200 nl	L1_MM6-BsaI	200 nl
Final volume	500 nl	Final volume	500 nl	Final volume	500 nl

Example of volumes dispensed by Echo in different L1-Loop reactions

7 **For L1 Loops, prepare MM for maximum of 6 parts as follows**:

	Volume for 1 reaction in 1 well in a 384 PCR plate	Volume for 96 reactions in a 384 PCR plate
Water	100 nl	9.6 ul
10x T4 ligase buffer (NEB)	50 nl	4.8 ul
1 mg/mL bovine serum albumin (NEB)	25 nl	2.4 ul
T4 DNA ligase at 400 U/µL (NEB)	12.5 nl	1.2 ul
10 U/µL BsaI (NEB)	12.5 nl	1.2 ul
Final volume	200 nl	19.2 ul

L1-MM6-BsaI

To prepare the MM always consider the volume you need for the plate reactions plus the dead volume of the MM solution in a PP plate.

Always prepare the MM in an eppendorf tube first, mix by pipetting and then transfer the MM to the PP-well. This is to ensure all components of the MM are well mixed.

8 **Generate a csv file to upload in the Labcyte cherry pick software with the following information:**

Source Plate Name	Source Well	Destination Well	Sample Name	Transfer Volume	Source Plate Type	Destination Plate Name	Destination Plate Type

Generation of the csv files can be done as more convinient, with an excel template or a phyton script, or ...
Previous to running and Echo experiment, make sure the information in the csv file is correct and the Echo is running what you intend to run. You can simulate the running at the Echo.

9 **Loop assembly using the Echo**:

Load csv file in the cherry pick.

Get LDV and PP source plates:
LDV plate: defrost, spin down and keept at RT.
PP plate: defrost, spin down, keep at RT. Prepare MM, spin and keep at RT.

Get the destination plates: skirted 384-PCR plates

Run the L1 or L2 Loop assemblies at the Echo.
If there are any exceptons, proceed to re-run those dispensations.

Seal the 384-PCR plate with a PCR foil seal and spin it down.

10

Place the 384-PCR plates at the termocycler and run the follwoing cycling conditions:

Assembly: 26 cycles of **37 °C** for **00:03:00** and **16 °C** for **00:04:00** .

Termination and enzyme denaturation: **50 °C** for **00:05:00** and **80 °C** for **00:10:00** .

11 **Transform the Loop assemblies with highly competent cells (10^8) as follows:**

Note: we use homemade Top10 HS higly competent cells.But any other ones higly competent will work as well.

Add 2 ul of cells to each well

Incubate **00:15:00** on ice

Heat shock at **42 °C** for **00:00:40** in a thermocycler

Incubate **00:02:00** on ice

Add 15 u of LB to each well

Seal the plate with a breath-easy sealing membrane

Incubate the plate at **37 °C** on a shaker for **01:00:00**

Plate(*) the ~ 18ul fo the reaction in LB agar plates with with either:
for L1 kanamycin 50 µg/mL and X-gal 40 ng/ mL
for L2 spectinomycin 100 µg/mL and X-gal 40 ng/ mL

Incubate plates **00:15:00** **O/N** at **37 °C**

All white colonies have an insert inside the AP vector. The efficiency of the assemnmbly is really high, and we normally just check 2 colonies.

(*) Alternatively, one can use the Echo to tranfer the transformed cells onto a plate. It needs transfering of the transformation to a PP plate and spotting with the Echo onto an omnitray.