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Library cloning

In 1 collection

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Abstract

This is the protocol for inserting the library gene of interest into the pre-barcoded vector created in the previous protocol (Barcoded Vector Cloning).

Materials

Materials

- Barcoded library plasmid
- Negative control from Golden Gate reaction
- SapI - 1,250 units **New England Biolabs Catalog #**R0569L
- rCutsmart Buffer (supplied with SapI restriction endonuclease)
- rSap
- Molecular Biology Grade Water **Thomas Scientific Catalog #**C001X09
- Rigid Strip 8-Strip Tubes **Thomas Scientific Catalog #**1148A28
- T4 DNA ligase buffer (supplied with T4 DNA ligase)
- T4 ligase **New England Biolabs Catalog #**M0202M
- Zymo DNA Clean & Concentrator Kit **Zymo Research Catalog #**D4014
- Qubit™ 1X dsDNA BR Assay Kit **Thermo Fisher Scientific Catalog #**Q33231
- Qubit dsDNA 1X HS Assay Kit (Thermo Fisher Scientific, Catalog # Q33266)
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog #**Q32856
- Ice
- NEB 10-beta Electrocompetent E.coli - 6x0.1 ml **New England Biolabs Catalog #**C3020K
- SOC (supplied with NEB cells)
- pUC19 positive control (supplied with NEB cells)
- Electroporation cuvettes, 1mm (Thomas Scientific, Catalog # 1181C48)
- Glass culture tubes (15ml)
- 500-mL Erlenmeyer flasks
- LB liquid media
- Carbenicillin **Gold Biotechnology Catalog #** C-103-5
- LB + Ampicillin agarose plates
- 1.7 ml microcentrifuge tube **Thomas Scientific Catalog #**1159M35
- Glass beads

Equipment

- C1000 Touch™ Thermal Cycler **Bio-Rad Laboratories Catalog #**1851148
- NanoDrop™ 2000 Spectrophotometer **Thermo Fisher Catalog #**ND-2000
- Qubit Fluorometer **Invitrogen - Thermo Fisher Catalog #**Q32866
- MicroPulser Electroporator **Bio-Rad Laboratories Catalog #**1652100
- Shaking incubator
- Standing incubator

50-uL reaction:

	A	B	C	D	E
	Reagent	**Vol for 1 reaction (uL)**	**Volume for 5 reactions (uL)**		
	H2O			Error	1.05
	DNA (1ug)			# reactions	5
	rCutsmart Buffer	5	26.25		
	SapI	1	5.25		
	Total volume:	50			

Ligation reaction:

	A	B	C	D	E	F	G	H
				Total samples #	4	-ctl	Err	1.05
	Component		Final conc.	1Rxn (uL)	Total			
	H2O			7.027266667	29.51452	14.5		
	T4 DNA ligase buffer		1X	2.5	10.5	2.5		
	T4 DNA ligase (2,000U/ul)			0.5	2.1	0.5		
	Plasmid (75ng/ul)	5400bp		1	4.2	1		
	SapI (10U/ul)		15	1.5	6.3	1.5		
	Sub-total			11.02726667	46.31452			
	Insert F1 (75ng/ul)	plasmid 4182bp/1656bp	174ng/ul (1:3mol ratio)	2.32	9.744	0		
	Insert F2 (75ng/ul)	plasmid 3144/621bp	131.7ng/ul (1:3 mol ratio)	1.756	7.3752	0		
	Insert F3 (75ng/ul)	Plasmid 4781/2258 bp	199ng/ul (1:3 mol ratio)	2.653333333	11.144	0		
	Insert 4 (50ng/ul)	Library 892bp	37.17ng/ul (1:3 mol ratio)	0.7434	3.12228	0		
	Total			20	#REF!			

Additional digestion reaction:

	A	B	C	D	E	F
			Total samples #	5	Err	1.05
	Component	Final conc.	1Rxn (uL)	Total		
	H2O		2.5	13.125		

	A	B	C	D	E	F
	rCutSmart Buffer		1	5.25		
	SapI (10U/ul)	10U	1.5	7.875		
	Sub-total		#REF!	#REF!		
	Total		5	#REF!		

Before start

- Ensure there are enough maxi-prep kits available to use.

Predigest the barcoded backbone

1 Set up a 50 µL reaction as outlined below:

	A	B	C	D	E
	Reagent	**Vol for 1 reaction (uL)**	**Volume for 5 reactions (uL)**		
	H2O			Error	1.05
	DNA (1ug)			# reactions	5
	rCutsmart Buffer	5	26.25		
	SapI	1	5.25		
	Total volume:	50			

2 Set the thermal cycler for the program below:

	A	B	C
	Step	**Temperature**	**Time (hh:mm:ss)**
	1	37°C	03:00:00
	2	65°C	00:20:00
	3	10°C	hold

Note

- Spike in additional 1ul of SapI enzyme every 60 mins, to each sample
- At the 2:30 mark, add 1ul of rSap- this is to dephosphorylate the 5' end to re-ligate the ends.

3 Run all digested samples on a 0.5% TBE gel to ensure complete digestion of the backbone.

- Also run 1 µg of original starting template as a visual reference for the supercoiled plasmid.

4 Gel extract the linearized product.

5 Use the Clean & Concentrator kit on the gel extraction product.

- To avoid issues with freeze-thaws, aliquot the cleaned, linearized product in small volumes.

Golden gate assembly of variant library inserts

4h

6

Note

This adds the variant library inserts to the pre-barcoded vector using SapI.

Set up reaction as outlined below:

6.1

Note

Here we normally use 1:3 nmol backbone to each insert ratio, below is an example with a backbone and 4 insert frag. please note three of the fragments are on the helper plasmid, thus the calculation is done on the whole helper plasmid.

6.2 Here is a **ligation calculator** by NEB to use if you don't have a formula set up.

6.3 Here is **NEB's general protocol for GG with SapI**.

6.4 Neg Ctl: Using the vector with no insert.

	A	B	C	D	E	F	G	H
				Total samples #	4	-ctl	Err	1.05
	Component		Final conc.	1Rxn (uL)	Total			
	H2O			7.0272666667	29.51452	14.5		
	T4 DNA ligase buffer		1X	2.5	10.5	2.5		
	T4 DNA ligase (2,000U/ul)			0.5	2.1	0.5		

	A	B	C	D	E	F	G	H
	Plasmid (75n g/ul)	5400bp		1	4.2	1		
	SapI (10U/ul)		15	1.5	6.3	1.5		
	Sub-total			11.02726 667	46.3145 2			
	Insert F1 (75n g/ul)	plasmid 4182b p/1656bp	174ng/ul (1:3 mol ratio)	2.32	9.744	0		
	Insert F2 (75n g/ul)	plasmid 3144/6 21bp	131.7ng/ul (1:3 mol rati o)	1.756	7.3752	0		
	Insert F3 (75n g/ul)	Plasmid 4781/2 258bp	199ng/ul (1:3 mol ratio)	2.653333 333	11.144	0		
	Insert 4 (50ng/ ul)	Library 892bp	37.17ng/ul (1:3 mol rati o)	0.7434	3.12228	0		
	Total			20	#REF!			

7 Set a thermocycler for the following program:

- Spike in 1ul of SapI every hour.

	A	B	C
	Step	**Temperature**	**Time (hh:mm:ss)**
	1	37°C	00:05:00
	2	16°C	00:05:00
	3	Go to step 1 for 15 total cycles	
	4	60°C	00:15:00
	5	4°C	hold

8 Set up additional digestion reaction as follows:

	A	B	C	D	E	F
			Total samples #	5	Err	1.05
	Component	Final conc.	1Rxn (uL)	Total		
	H2O		2.5	13.125		
	rCutSmart Buffer		1	5.25		
	SapI (10U/ul)	10U	1.5	7.875		
	Sub-total		#REF!	#REF!		
	Total		5	#REF!		

9 Incubate at 37 °C for at least 02:00:00, or Overnight. 4h

- Spike in SapI every hour.

10 Purify the product with the Zymo Clean & Concentrator kit.

- Pool 2 sample reactions (40 µL) for 4 columns.

11 Measure the final eluate with Nanodrop to look for a normal purity curve.

- 260/280 ratio should be:

12 Quantify the product concentration with Qubit BR assay.

- The final eluate should be >5ng/uL for the samples and very low for the negative control.

Transformation with electro-competent cells 17h 56m

13 **Prepare cells for transformation**

13.1 Warm SOC to 37 °C.

13.2 Chill empty cuvettes On ice for 00:15:00. 15m

13.3 Thaw NEB electrocompetent cells (100 uL/tube) on ice for approximately 00:05:00. 5m

13.4 To chilled cuvettes, add 26 µL of thawed cells.

- Pipette to first stop only - do not create air bubbles as this will cause the electroporation to fail.
- Can use long gel-loading tips to do this more easily.

13.5 Add 1 µL - 2 µL of cleaned DNA from golden gate reaction and controls to the corresponding cuvette containing cells.

- Pipette to first stop only - do not create air bubbles.
- Do not add more than 3 uL of plasmid.

13.6 Flick the cuvette gently, then lightly tap on the bench. Place the cuvette back On ice .

13.7 Incubate On ice for 00:30:00 . 30m

14 Prepare for recovery

14.1 While the cells are incubating On ice , prepare glass recovery tubes with 900 µL SOC each, and warm to 37 °C .

14.2 Set the MicroPulser Electroporator to Manual for 2.0 ms.

- The electroporator is automatically set for 6 kV.

14.3 Prepare six overnight growth Erlenmeyer flasks with 300 mL of LB + 30 µL carbenicillin for each sample.

- Use 500 mL flasks for enough air circulation.

14.4 Label the LB + ampicillin dilution plates for each sample. Place in 37 °C after labeling to pre-warm.

- For Negative Golden Gate control, need 2 dilution plates
- For library and pUC19, need 3 dilution plates each.

15 Electroporation

15.1 Starting with the negative control cuvette, wipe the outside of the cuvette dry to remove condensation and ice.

15.2 Place the cuvette in the electroporator, ensuring the metal on the cuvette makes contact with the electrodes, and press the Pulse button.

- If the electroporator makes a loud noise and displays “Arc” on the digital readout, the sample has failed and is no longer good.

15.3 Remove the cuvette from the electroporator and place back On ice .

15.4 Repeat with all cuvettes prepared.

16 Sample recovery

16.1 Remove the glass recovery tubes containing the SOC from 37 °C .

16.2 Transfer the cells from each cuvette to the glass recovery tubes.

16.3 From the warmed bottle of SOC, add 100 µL to wash the electroporation cuvette, then transfer from the cuvette to the corresponding glass recovery tube. Repeat this for all samples.

- Long gel-loading tips may be helpful for sample recovery from the cuvettes.

16.4 Place the glass recovery tubes into the shaking incubator for 01:00:00 at 37 °C . 1h

17 Prepare dilution tubes and plates

17.1 As the cells are recovering, prepare microcentrifuge tubes for serial dilution of the library and controls. We recommend to start with a bottleneck scheme by dividing the 1ml recovery culture to 6 portions: 500 µL , 250 µL , 125 µL , 2x 50 µL , and 25 µL .

- For each portion, prepare 4 tubes (D0-D3) with the listed volume of LB. Label three plates (D1-D3) for each bottleneck portion (total of 18 plates).

	A	B	C	D	E	F
		Starting (S)	D0	D1	D2	D3
	Volume	300ml	300ul fro m S	100ul of D0 + 900 ul of LB	100ul of D0 + 900 ul of LB	100ul of D0 + 90 0ul of LB
	Plating		No plating	100ul	100ul	100ul

	A	B	C	D	E	F
	Dilution		0.736111111	1:30,000	1:300,000	1:3,000,000

17.2 For Puc, prepare two tubes and plates as the following Negative control and H_2O.

	A	B	C	D
		Starting (S)	D0	D1
	Volume	1ml	20ul from S +180 ul of LB	100ul of D0 + 900ul of LB
	Plating		100ul	100ul
	Dilution		1: 100	1:1,000

17.3 For Negative control and H_2O, plate 100 mL from the 1 mL recovery culture to the respective plates.

17.4 3m

Note

Prepare bottleneck for overnight growth and plating dilution plates.

- After the 1 hour recovery, add the appropriate portion(as below) from the 1 mL culture to the respective 500-mL flask based on the bottlenecking scheme.
- Place all 500 flasks in the 37 °C shaking incubator for 00:03:00 to ensure it is well mixed. No need to prepare overnight growth flasks for Puc19, negative control, and H_2O controls.

	A	B	C	D	E
		D0 (1:1000)	D1 (1:30,000)	D2 (1:300,000)	D3 (1:3,000,000)
	500ul				
	250 ul				
	125ul				
	50ul-1				
	50ul -2				
	25ul -1				

17.5 Mix each dilution well. Plate each dilution according to the dilution scheme above. Incubate Overnight at 37 °C. 3m

17.6 Incubate Overnight growth flasks in 30 °C for 14:00:00 - 16:00:00 . 1d 6h 3m

Estimating barcode coverage for bottlenecking

18 Count colonies on dilution plates to calculate the approximate variant to barcode coverage

For examples:
Library X : 205 x 22 variant changes = 4510 aa variants. We want to have to pick the flask that produces between 45,100 -135,300 colonies. This indicates between 10-30x coverage over the variants.

Note

Best to average all three dilution plates for each bottleneck portion to ensure the most accurate estimation proceed to the maxi preps if the coverage is good.

	A	B	C	D	E
		D0 (1:1000)	D1 (1:10,000)	D2 (1:100,000)	D3 (1:1,000,000)
	500ul				
	250 ul				
	125ul				
	50ul				
	50ul -1				
	25ul -2				