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Barcode plasmid library cloning

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

Protocol for cloning Time Machine barcode plasmid library.

Materials

MATERIALS

- Gibson Assembly Master Mix - 10 rxns **New England Biolabs** **Catalog #**E2611S
- Electroporation Cuvette 1mm **Bio-Rad Laboratories** **Catalog #**1652089
- Monarch® PCR & DNA Cleanup Kit (5 µg) **New England Biolabs** **Catalog #**T1030
- LB plates with 100 µg/ml ampicillin
- LB Broth with 100ug/mL ampicillin **Gibco - Thermo Fisher Scientific** **Catalog #**244620
- Lucigen Endura electrocompetent cells **Lucigen** **Catalog #**60242
- ssDNA barcode template **IDT**
- Linearized vector backbone
- EndoFree Plasmid maxiprep kit **Qiagen** **Catalog #**12362

Gibson assemnbly

1 Perform Gibson assembly usiing 100ng linearized vector and 5:1 molar excess of ssDNA template per 20 microliter reaction.

1.1 Pre-heat thermal cycler to 50ºC with lid set to 65ºC.

50 °C Pre-heat thermal cycler

1.2 Combine 100ng linearized vector with ~21.7 fmol ssDNA template (1.08 microliters of 100nM oligo) to a final volume of 10 microliters in nuclease free water. In parallel, perform a negative control reaction with plasmid and nuclease free water without the ssDNA insert. Keep on ice.

100 ng Linearized vector

1.08 µL ssDNA oligo (160nt)

10 µL Final volume in NF water

1.3 Thaw Gibson assembly master mix on ice then add 10 microliters to plasmid plus insert. Pipette mix then spin down if needed.

10 µL Gibson Assembly master mix

1.4 Incubate on thermal cycler at 50ºC for 1 hour.

50 °C 01:00:00 incubation

2 Purify Gibson reaction using PCR clean-up column according to manufacturer's protocol eluting in 20 microliters of

2.1 Add 40 microliters of DNA binding buffer to 20 microliters of Gibson assembly.

40 µL DNA binding buffer

20 µL Gibson assembly

2.2 Re-load eluant and re-spin.

2.3 Add 200 microliters DNA wash buffer. Make sure ethanol has been added to wash buffer before use.

200 µL Wash buffer

2.4 Centrifuge at 12,000 x g for 1 minute.

12000 x g

00:01:00

2.5 Repeat wash and centrifugation (steps 2.4 and 2.5)

2.6 Transfer column to a new eppendorf or collection tube then centrifuge at 12,000 x g for 2 minutes to dry column

12000 x g 00:02:00

Note

Sometime residual liquid can collect on a lip inside the column. It may be helpful to rotate the column 180º after a 1 minute spin, then repeat another 1 minute spin to get rid of this liquid.

2.7 Add 10 microliter of nuclease free water pre-heated to 50ºC to column and incubate for ≥ 2 minutes

10 µL nuclease free water 50 °C Pre-heat water 00:02:00 Incubate

2.8 Centrifuge at 12,000 x g for 1 minute to elute

12000 x g 00:01:00

2.9 Optionally nanodrop the recovered DNA. Expected concentration is 10-15ng/uL.

Electroporation

3 Elecroporation of competent cells

3.1 Pre-warm Lucigen recovery media to 37ºC

37 °C Pre-warm recovery media

3.2 If using Biorad Gene Pulser, pre-chill cuvettes to 4ºC.

4 °C Pre-chill electroporation cuvettes

3.3 Thaw Lucigen Endura electrocompetent cells on ice. Each DUO vials contains 50uL of cells, enough for 2 electroporations. Use one reaction's worth of cells to electroporate the negative control Gibson assembly.

3.4 Pipet vial of electrocompetent cells into 2 25 microliter aliquots.

3.5 Add 2 microliters of cleaned Gibson assembly to 25 microliters of electrocompetent cells.

2 µL Gibson assembly per 25 µL electrocompetent cells

3.6 Transfer electrocompetent cells to pre-chilled cuvette. Keep on ice.

3.7 Using the GenePulserXCell, electroporate with the following manual pulse setting: PL - 25msec, C - 10uF, PC- 600Ohms, V-1800

Note

Pulse length - 25msec, Capacitance - 10 microFaraday, Resistance - 600 ohms, Voltage - 1800 Volts.

Note

Expected time constant around 5-6 msec.

3.8 Immediately add 975 microliters of pre-warmed recovery media to cuvette then transfer to clean eppendorf or vial.

3.9 Incubate on shaker at 250 rpm at 37ºC for 1 hour.
37 °C 01:00:00 On shaker at 250rpm

3.10 Plate dilutions of electroporated cells for plating and calculating electroporation efficiency.
Prepare first dilution by adding 10 µL electroporated cells to 990 µL LB Broth
Prepare second dilution by adding 100 µL First dilution to 900 µL LB broth
Plate 100 µL first dilution on pre-warmed LB/ampicillin agar plate for a 1:1000 dilution plate.
Plate 100 µL second dilution on pre-warmed LB/Ampicllin agar plate for a 1:10,000 dilution plate.

Expected result

After incubating the plates overnight, expect to see >1,000 colonies on the 1:1000 dilution plate and >200 colonies on the 1:10,000 plate.

3.11 Add the remainder of the electroporated cells to 150mL LB broth containing 100ug/mL ampicillin. Incubate 12-14 hours on shaker at 32ºC.
150 mL LB broth
32 °C
12:00:00 - 14:00:00 shaker

3.12 Pellet the overnight culture by centrifugation in 50mL falcon tubes then store pellets at -20ºC or proceed immediately with maxiprep.

6000 x g | 00:15:00 | 4 °C Pellet e. coli cultures.

-20 °C Store e. coli pellets

Note

If centrifuge capable of 6,000 x g is unavailable, can pellet at ≥3,000 x g.

4 PCR screen colonies to confirm barcode insertion.

5 Maxiprep barcode plasmid library.