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# Barcoded vector cloning

In 1 collection

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## Abstract

This is the protocol for inserting barcodes to the N- and C- vector.

## Materials

**Materials:**

- Barcode sequence as described above in “Barcode sequence design”
- Forward primer and degradative reverse primer as described above in “Barcode sequence design”
- Esp3I - 1,500 units **New England Biolabs Catalog #**R0734L
- BsmBI-v2 (New England Biolabs, Catalog # R0739)
- NEBuffer r3.1 (supplied with BsmBI-v2 restriction endonuclease)
- 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)
- Corning™ Untreated 245mm Square BioAssay Dishes **Fisher Scientific Catalog #**07-200-600
- 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

**qPCR reaction: 25ul/rxn**

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | | Total samples # | 4 | Err | 1.05 |
| | Component | Final conc. | 1Rxn (uL) | Total | | |
| | H2O | | 8.5 | 35.7 | | |
| | Q5 MM (2X) | 1X | 12.5 | 52.5 | | |
| | SYBR (100X) | 2x | 0.5 | 2.1 | | |
| | template oligo (10ng/ul) | | 1 | 4.2 | | |
| | Sub-total | | 22.5 | 165.4 | | |
| | | | | | | |
| | F primer (10uM) | | 1.25 | 5.3 | | |
| | R primer (10uM) | | 1.25 | 5.3 | | |
| | Sub-total | | 2.5 | 10.5 | | |
| | Total | | 25 | 183.75 | | |

**Golden gate reactions:**

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | | Total samples # | 7 | Err | 1.05 |
| | Component | Final conc. | 1Rxn (uL) | Total | | |
| | H2O | | Up to 25 uL | | | |
| | T4 DNA ligase buffer | 1X | 2.5 | 18.4 | | |
| | T4 DNA ligase (2,000U/ul) | | 0.5 | 3.7 | | |
| | Plasmid (100ng/ul) | 250 ng | 2.5 | 9.849 | | |
| | Esp3I (10U/ul) | 30U | 2 | 14.7 | | |
| | Sub-total | | 21.2 | 155.82 | | |
| | Barcode amplicon Insert (ul) | 1:3 mol ratio | Variable | Variable | | use H2O instead for Neg. ctl |
| | Total | | 25 | #REF! | | |

**Digestion mix:**

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | | Total samples # | 8 | Err | 1.05 |
| | Component | Final conc. | 1Rxn (uL) | Total | | |
| | H2O | | 3 | 25.2 | | |
| | rCutSmart Buffer | | 1 | 8.4 | | |

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | BsmBI-v2 (10U/ul) | 10U | 1 | 8.4 | | |
| | Total | | 5 | #REF! | | |

## Before start

- Ensure there are enough maxi-prep kits available to use.
- Prepare 2-3 of 245 mm LB plus ampicillin/Carbenicillin agarose plates. Each plate should have 350-400 mL of agarose in LB with 1:1000 of ampicillin/Carbenicillin

## Generate barcode amplicon

1 qPCR reaction: 25ul/rxn

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | | Total samples # | 4 | Err | 1.05 |
| | Component | Final conc. | 1Rxn (uL) | Total | | |
| | H2O | | 8.5 | 35.7 | | |
| | Q5 MM (2X) | 1X | 12.5 | 52.5 | | |
| | SYBR (100X) | 2x | 0.5 | 2.1 | | |
| | template oligo (10ng/ul) | | 1 | 4.2 | | |
| | Sub-total | | 22.5 | 165.4 | | |
| | | | | | | |
| | F primer (10uM) | | 1.25 | 5.3 | | |
| | R primer (10uM) | | 1.25 | 5.3 | | |
| | Sub-total | | 2.5 | 10.5 | | |
| | Total | | 25 | 183.75 | | |

1.1 Thermocycling in a qPCR machine:

| | A | B | C |
|---|---|---|---|
| | **Step** | **Temperature** | **Time (hh:mm:ss)** |
| | 1 | 98°C | 00:00:30 |
| | 2 | 98°C | 00:00:10 |
| | 3 | 55-70°C | 00:00:10 |
| | 4 | Go to step 1 for 15 total cycles | |
| | 4 | 72°C | 00:15:00 |
| | 5 | Got to step 2 | Pull samples in logarithmic phase |

2 Purify with Zymo Clean & Concentrate with pooling 4 reactions together for one column: elute the column with 14 µL $H_2O$. This is counting the volume needed for qubit and nanodrop.

3 Quantify concentration of the final elution with Qubit and Nanodrop.

Note

- Use the concentration from Qubit for downstream steps.
- option : use tapestation to visualize the amplicon and quantify concentration.

## Generating barcoded vectors using golden gate assembly

4 Setting up golden gate reactions:

Note

We recommend setting up 7 sample reactions and 1 negative control with no insert.

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | | Total samples # | 7 | Err | 1.05 |
| | Component | Final conc. | 1Rxn (uL) | Total | | |
| | H2O | | Up to 25 uL | | | |
| | T4 DNA ligase buffer | 1X | 2.5 | 18.4 | | |
| | T4 DNA ligase (2,000U/ul) | | 0.5 | 3.7 | | |
| | Plasmid (100ng/ul) | 250 ng | 2.5 | 9.849 | | |
| | Esp3I (10U/ul) | 30U | 2 | 14.7 | | |
| | Sub-total | | 21.2 | 155.82 | | |
| | Barcode amplicon Insert (ul) | 1:3 mol ratio | Variable | Variable | | use H2O instead for Neg. ctl |
| | Total | | 25 | #REF! | | |

4.1 Aliquot 25 µL per reaction to 8-well strip tube.

4.2 Set the thermal cycler for the program below:

| | A | B | C |
|---|---|---|---|
| | **Step** | **Temperature** | **Time (hh:mm:ss)** |
| | 1 | 37°C | 00:05:00 |

| | A | B | C |
|---|---|---|---|
| | 2 | 16°C | 00:05:00 |
| | 4 | Go to step 1 for 20-30 total cycles | |
| | 5 | 60°C | 00:15:00 |
| | 6 | 85°C | 00:15:00 |
| | 7 | 4°C | hold |

5 Make digestion mix:

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | | Total samples # | 8 | Err | 1.05 |
| | Component | Final conc. | 1Rxn (uL) | Total | | |
| | H2O | | 3 | 25.2 | | |
| | rCutSmart Buffer | | 1 | 8.4 | | |
| | BsmBI-v2 (10U/ul) | 10U | 1 | 8.4 | | |
| | Total | | 5 | #REF! | | |

5.1 Aliquot 5 µL to each golden gate reaction.

5.2 Set the thermal cycler for the program below:

| | A | B | C |
|---|---|---|---|
| | **Step** | **Temperature** | **Time (hh:mm:ss)** |
| | 1 | 55°C | At least 2 hours |
| | 2 | 80°C | 00:15:00 |
| | 4 | Hold at 4C | |

6 Purify with Zymo Clean & Concentrator -5 kit: pooling 3-4 reactions for each column with final elute to 10 µL .

7 Measure the final elute with Nanodrop to look for normal purify curve, and quantify with Qubit: final reaction elution should be > 5ng/ul. The negative control should be < 5ng/ul

## Transformation with electro-competent cells and plating

1d 5h 50m

8

Note

- Depending on the final Golden gate concentration and volume, aim for ~15- 20ng of DNA per 25ul of NEB-beta for each sample reaction.
- Keep total volume of input DNA <3ul to avoid cell death. Use 1ul for controls: golden gate negative control, Puc 19 control, and $H_2O$ control.
- For each vector to be barcoded, aim for at least 4x 10^8 total barcodes. This requires plating 2-3 of 245mm plates.
- Each 245 mm plate can hold no more than 4 electro-competent reactions recovered in no more than 4 ml of SOC.

9 Pre-chill cuvettes On ice for at least 00:15:00 . 15m

10 Aliquot 875 µL of SOC media for each electroporation reaction to glass tubes. Pre-warm the tubes with media and an additional bottle of SOC in 37 °C .

11 **Prepare cells:** 5m

Thaw vials NEB electrocompetent cells (100ul/tube) On ice -immediately before the transformation. The cells start to die after ~ 00:05:00 .

12 Add 25 µL thawed cells to each cuvette by tilting each cuvette towards you for easier pipetting. Stop at the 1st pipette stop to avoid injecting air bubbles to the cuvette. Air will cause your samples to “arc”.

13 Add DNA to each sample to its cuvette into the cells with the cuvette tilted. Again, stop at the 1st pipette stop to avoid injecting air bubbles to the cuvette. Air will cause your samples to “arc”.

14 Flick and tap cuvette after DNA is added with fingers, then lightly tap cuvettes a couple of times on the bench to mix. Put it back On ice before proceeding to the next samples.

15 Chill all sample On ice for 00:30:00 . 30m

16 **Preparation for recovery:**

While cells are chilling On ice .

Set up the machine: select for Manual at 2.0 ms- this will automatically set for 6 kV.

17 **Electroporation:**

Wipe cuvette dry with kimwipe to remove condensation and ice.

18 Start with the "$H_2O$ control". Electroporate each sample; make sure it makes connection to the metal. If a sample "arc", it is no longer good.

19 Put each On ice before recovery them.

20 **Sample recovery:**

Take the tubs with SOC from 37 °C .

21 Transfer each of the cell + DNA (~26-28u) to the labeled tubes. Put each cuvettes back On ice .

22 Wash each curettes with 100 µL of warm SOC and transfer to recovery tubes.

23 Put the recovery culture at 37 °C for 01:00:00 with shaking. 1h

24 **Preparing Dilution for plating:**

Prepare 4 tubes (D0- D3) for each sample for a serial dilution with LB as below. Mix well.

Note

Sample reactions can be combined to represent the larger plate plating with 2 mL - 3 mL of recovery culture. Then modify D0 dilution accordingly and dilution factors.

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | Starting (S) | D0 | D1 | D2 | D3 |
| | Volume | 1ml | 10ul of S + 990 ul of LB | 100ul of D0 + 900ul of LB | 100ul of D0 + 900ul of LB | 100ul of D0 + 900ul of LB |
| | Plating | | No plating | 100ul | 100ul | 100ul |
| | Dilution | | 1:100 | 1:10,000 | 1:100,000 | 1:1,000,000 |

25 For each sample and Puc 19, label three 10cm LB + amp plates (D1-D3) and plate 100 µL from the D1-D3 corresponding tubes.

- For negative control and $H_2O$, plate 100 µL from the 1 mL recovery culture.

26 Spread the plated culture with beads.

27 Place the plates in 37 °C for Overnight /faster growth. 8h

28 **Plating the large plate:**

Pipette 2 mL - 3 mL of recovery culture to each large LB+Carb plate, use the roller to spread the cell cultures. Leave the lids ajar slightly to dry the surface.

> **Note**
>
> No need to plate controls on the large plates.

28.1 Put all plates in 30 °C for 16:00:00 - 20:00:00 incubation . 20h

28.2 Chill one tube of 50ml LB/plate in 4 °C for harvesting the next day.

29 **Harvesting:**

The next day, count the colonies on  D2 and D3 dilution plates to estimate total colonies on each large plate.

**Note**

For 2 x 10^8 barcodes, there should be 200 colonies on the D3 plates.

29.1 The large plates must be harvested and prepped with a preferred plasmid Maxi prep kit immediately.