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# OG1RF_transposon_mutant_library_protocol

Elizabeth Fozo[1]
[1]In-house protocol

1 Works for me This protocol is published without a DOI.

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ABSTRACT

**Generation of Transposon Mutant Library**

PROTOCOL CITATION

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MATERIALS TEXT

**Transformation recipes:**

Electroporation Buffer (EB) 50 mL
- 0.5 M sucrose 8.56 g
- 10% glycerol 5 mL 100% stock bottle
- Bring volume to 50 mL with dH2O.
- Autoclave or filter sterilize.

Lysozyme Solution (LS) 50 mL
- 10 mM Tris pH 8.0 0.5 mL 1 M
- 20% sucrose 10 g or 40 mL 25%
- 10 mM EDTA 1 mL 0.5 M pH 8.0
- 50 mM NaCl 0.5 mL 5 M
- Bring volume to 50 mL with dH2O.
- Autoclave or filter sterilize.

STHB 100mL
- 0.5M Sucrose 17.115g
- THB powder 3g
- Bring up to 100 mL with dH2O.
- Autoclave.

DISCLAIMER:

DISCLAIMER: THIS WORK IS IN PROGRESS. IT IS FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

ABSTRACT

## Generation of Transposon Mutant Library

BEFORE STARTING

Refer to Materials for Transformation recipes

Antibiotics/antibacterial peptides notes:

- Nisin is diluted in sterile water and is not filter-sterilized.
- Chloramphenicol is diluted in methanol. (can be diluted in ethanol, but for this protocol, methanol was used)
- Erythromycin is diluted in methanol. (can be diluted in ethanol, but for this protocol, methanol was used)
- Fusidic acid is diluted in sterile water and is filter-sterilized.

Transform transposase-carrying plasmid (pCJK55) into E. faecalis strain of interest.

1 Inoculate 5-10 mL BHI with strain of interest (can use fusidic acid at 25 µg/mL) and incubate overnight at 37°C.

2 Dilute o/n culture 1:10 in morning in 100 mL THB (or BHI).

3 Incubate at 37°C until OD at 600 nm reaches 0.5 -1.0.

4 During cell incubation, chill two 50 mL conicals and two 1.5 mL Eppendorf tubes on ice, thaw 1 mg/mL lysozyme aliquot.

5 Aliquot cells into the two chilled 50 mL conicals.

6 Chill cells on ice for 15-20 minutes. Pellet the cells at 6,000 rpm 10 min.

7 While cells are chilling, add lysozyme to 2.5 mL of lysozyme solution to make a 25 µg/mL solution of lysozyme.

8 Resuspend pellet in 2 mL ice cold water. Aliquot to two chilled 1.5 mL Eppendorf tubes. Pellet cells.

9 Resuspend each pellet (there should be 4 at this point) in 500 µL lysozyme solution.

10 Incubate cells in 37°C water bath for 20 min.

11

**Everything is always on ice from this point forward!!!**

Chill all electrocuvettes needed; all Eppendorf tubes needed (depends on the number of transformations you are doing), and aliquots of pCJK55. Do NOT chill tubes for recovery or recovery media.

Check at step 14 to determine how many chilledEppendorfsyou need

12 Pellet cells. Wash with 0.5 mL ice-cold electroporation buffer 3x.

13 Resuspend each pellet (four pellets) in 150 µL electroporation buffer. Pool aliquots of cells.

14 Aliquot 50 µL cells to chilledEppendorfsfor transformations (number of tubes depends on number of transformations). Aliquot 100 µL remaining cells to chilledEppendorfsfor freezing for future transformations.

15 Cells to be frozen can be stored at -80°C. No more than one freeze/thaw cycle per aliquot. Efficiency decreases slightly after freezing.

16 Mix ~100 ng pCJK55 with cells to be transformed. Transfer to chilled electro cuvette and electroporate (setting = EC2); immediately add 1 mL STHB to electro cuvette after the pulse is completed, wash cells out of electro cuvette, and transfer to an Eppendorf tube. The time constant should be ~5.0. Proceed even if the time constant is not 5.0 or if arcing occurs.

17 Recover cells in a 37°C water bath for ~2-4 hours.

18 Plate 50 µL undiluted cells on BHI + 10 µg/mL Erm. Pellet remaining volume and resuspend in 100 µL STHB. Plate 50 µL resuspended colonies. Save the rest on your benchtop overnight.

19 Incubate plates at 37°C until colonies appear (1-2 days).

## Generate transposon mutant library

20 Start 5 mL overnights of transformants holding pCJK55 and of donor plasmid carrying transposable element (CK111 + pCF10-101 + pCJK72) in small test tubes at 3:00 p.m. Incubate overnight at 30°C.

Note that the tubes are: Fisherbrand16 x 100 mm borosilicate glass (14-961-29).
Note that donor and recipient o/ns are supplemented with 10 µg/mL Erm.

21 On the following morning, in the same size of tubes, subculture 10 mL (1:20 dilutions).
- Donors: 0.5 mL of o/n mixed with BHI supplemented with 1 µg/mL Erm
- Recipients: 0.5 mL of o/n mixed with BHI supplemented with 1 µg/mL Erm and 25 ng/mL nisin

22 Incubate cultures at 30°C for one hour and 45 min.

23 Mix donors and recipients, controls in Eppendorf tubes:
- Donor only mix: 100 µL donor + 900 µL BHI
- The recipient only mix: 100 µL BHI + 900 µL recipient
- Transconjugantmix(es): 100 µL donor + 900 µL recipient

24 Centrifuge 1 min 13k rpm (small centrifuge). Remove 850 µL supernatant. Plate on BHI + 25 ng/mL nisin

25 Incubate at 30°C for ~20 hours.

26 After ~20 hours, scrape all plates (there should be lawns on every plate) with 1 mL BHI + 2mM EDTA. Pipette into 2 mL Eppendorf tube, and repeat scraping with an additional mL BHI + 2mM EDTA.

27 Centrifuge mixture for 1 min at 13k RPM.

28 Remove supernatant and resuspend in 1.8 mL BHI + 2mM EDTA + 30% glycerol.

29 Split cells into two 1 mL aliquots, labeled A and B. Make dilutions from each of these aliquots:
- Donors and recipients only – dilute out to 10-2. Transconjugants– dilute out to 10-3.
- 10-1– 100 µL stock + 900 µL BHI
- 10-2– 100 µL 10-1+ 900 µL BHI
- 10-3– 100 µL 10-2+ 900 µL BHI

30 Plate dilutionsfrom aliquots A and Bon large 150 mm x 15 mm round plates containing BHI + 10 µg/mL chloramphenicol + 25 µg/mLfusidicacid + 150 µg/mL X-gal:
- Donor only: plate 100 µL of the 10-2dilution
- Recipient only: plate 100 µL of the 10-2dilution
- Transconjugants: plate 100 µL of the 10-2dilution, 100 µL of the 10-3dilution.
- Note that large plates contain~50-60 mL agar(500 mL should make about 10 plates).

31 Freeze the 1 mL stocks and the dilutions at -80°C.

32 Incubate the plates at 37°C until colonies are countable (~20-30 h).

33 Count blue/white colonies on plates (~3000 colonies/plate is a good number). Calculate the percentage of blue/white colonies. Ideally, you want under 4% blue colonies.

- Donor only and recipient only plates should have no colonies.
- Determine from the percentage of blue colonies whether you should continue. If continuing, calculate the number of plates you will need to reach approximately the size of library you wish to have (if each plate has 3000 white colonies, you will need 34 or so plates to reach a library of 100,000 mutants.)

34 Patch 10 white colonies on BHI + 10 µg/mL chloramphenicol + 25 µg/mL fusidic acid + 150 µg/mL X-gal. Use the same colony to inoculate o/n cultures in BHI + 10 µg/mL chloramphenicol for genomic DNA isolation.

Genomic DNA to be used to perform Southern blotting to determine the distribution of transposons within the genomic DNA (distribution should be random and there should only be one transposon insertion/clone).

35 Scrape each transconjugant plate with 3 mL BHI + 10 µg/mL chloramphenicol + 25 µg/mL fusidic acid + 10% glycerol. Place in 15 mL plastic conical. Scrape plate again with 2 mL BHI + 10 µg/mL chloramphenicol + 25 µg/mL fusidic acid + 10% glycerol, and add to the same conical.

36 Spin down. Resuspend in 0.5 mL BHI + 10 µg/mL chloramphenicol + 25 µg/mL fusidic acid + 10% glycerol.

37 In a 96-well plate, make dilutions out to 10-7.
- In the top well, add 100 µL of the cells.
- In the seven wells below, add 90 µL BHI.
- Make serial dilutions down to 10-7.

38 Spread 10 µL of each dilution on a plate/lane (can use a multi-channel pipette and drip down 10 µL, allow to stream down the plate and dry). Do not allow streams to run together or all the way to the bottom of the plate.

- Square plates are preferable, but this can be done with circle plates which will hold fewer lanes (~4 lanes).
- Each dilution does not have to be plated; dilutions from 10-5-10-8 can be done; likely lower dilutions will have too many colonies to count.

39 Incubate plates at 37°C. Count plates. Multiple counts can be done; if you suspect more smaller colonies will appear after further incubation, you can do an initial count and place back in the incubator, then count again to get as accurate of a count as you can. For 10 µL lanes, there should be at least 30 clones to count; otherwise you are likely not getting an accurate count.

40 Calculate CFUs from the counts.

To make pooled libraries, make 150 mm x 15 mm round plates containing BHI + 10 µg/mL chloramphenicol + 25

41 µg/mL fusidic acid + 150 µg/mL X-gal (number depends on calculations in step 14).

42 Plate transconjugant dilutions as in step 11; use appropriate dilution (likely 100 µL of the 10-3 dilution). Incubate plates at 37°C. Count a few plates to ensure approximately the same number of colonies is obtained/plate as expected and that the blue:white colony ratio is the same (for example, 3000 colonies, with <4% blue colonies).

43 Scrape plates into varying amounts of BHI + 10 µg/mL chloramphenicol + 25 µg/mL fusidic acid + 10 % glycerol and create aliquots (this will need to be decided upon based on the number of plates).

44 Store aliquots at -80°C.