

Title: Generation of Large-Scale Transposon Mutant Libraries in *Brucella abortus*[†]
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MATERIALS

Brucella abortus culture

Or any strain that is a suitable recipient by conjugation. Adjust culture conditions accordingly.

Donor bacterial strain containing selectable transposon on vector

Escherichia coli MFD Δ dap strain recommended(10, 11)

2YT liquid media (recipe below)

2YT with 0.3 mM D-amino-pimelic acid (DAP)

2YT agar plates – 100 mm

2YT + antibiotic selective plates (e.g. kanamycin at 50 μ g/ml) – 100 mm and 150 mm

Selection antibiotic stock: e.g. kanamycin

Sterile Erlenmeyer flasks

10 ml sterile culture tubes

50 ml sterile conical tubes

1.5 ml sterile microcentrifuge tubes

90 mm diameter sterile 0.2 μ m polyethersulfone filters

25 mm diameter sterile 0.2 μ m polyethersulfone filters

Metal forceps

Di-methyl sulfoxide (DMSO)

2X Yeast Tryptone Liquid Medium

16 g bacto-tryptone

10 g bacto-yeast extract

5 g NaCl

Add to 950 ml deionized H₂O; pH to 7.0 with NaOH.

Adjust volume to 1 liter;

Sterilize by autoclaving.

For **solid medium**, add 15 g agar per liter

WARNING

Please take all appropriate precautions for biocontainment of *Brucella abortus* and adjust for BSL3 safety practices with regard to materials and steps.

METHODS

Note: this protocol is optimized to generate a large-scale transposon insertional mutant library of approximately 1 million cells. Scale accordingly to your needs.

Day 0 references the start of library generation methods. Days prior refer to optimal preparation steps to coordinate timing of protocol.

Day -5

1. Streak from frozen stock of *B. abortus* onto 2YT media plate. Incubate at 37°C for 3 days.

Day -2

1. Inoculate 1 colony of *B. abortus* into 1 ml of liquid 2YT media. Incubate, shaking, at 37°C overnight.
2. Streak from frozen stock of the donor mating *E. coli* strain onto agar plate containing selection antibiotic for plasmid retention. Incubate at 37°C overnight.

Day -1

1. Culture of *B. abortus* should be turbid. Transfer 1 ml of culture into 50 ml of 2YT media in a large (500 ml or 1L) sterile capped flask. Incubate while shaking at 37°C overnight.
2. Inoculate 1 colony of donor mating *E. coli* strain into 50 ml LB media containing appropriate concentration of selection antibiotic in a sterile capped flask. Incubate while shaking at 37°C overnight.

Note: This protocol references an diaminopimelic acid (DAP) auxotrophic donor strain, which eliminates the need to select for *Brucella* with antibiotics. Steps that require DAP supplementation are indicated in this protocol.

Day 0

1. Use proper aseptic technique to transfer 4 ml of turbid overnight *B. abortus* culture into a culture tube. Repeat for a total of 4 tubes, or 16 ml total.
2. Similarly transfer 4 ml of turbid overnight *E. coli* culture into culture tubes, generating 4 tubes, 16 ml total.
3. Centrifuge the tubes at 3000 rpm x 10-15 minutes.
4. Discard the supernatant.
5. Add 200 µl of mating media* (2YT) with 0.3 mM DAP to each culture tube, and vortex to resuspend cells.

- a. *The most common media is LB, but supplemented defined media can work too, as long as both donor and recipient strains can both grow on it. Note that S17 strains typically require a source of thiamine.
6. Consolidate all *B. abortus* cultures and *E. coli* cultures each to a single tube respectively.
 - a. 4 tubes = ~0.8 ml pool.
7. Combine equal volumes of resuspended WT culture and *E. coli* culture together into a sterile culture tube. (e.g. 1 or all 2 ml of each culture) Gently mix. This is the mating mixture.
 - a. Leave at least 50 μ l remaining of *B. abortus* and *E. coli*-only (unmixed) cell cultures for controls
8. Using sterile flamed forceps, place sterile 25 mm 0.2 μ m polyethersulfone filters onto mating media agar containing 0.3 mM DAP.
 - a. Seven should be sufficient to produce > 1 million conjugants.
9. Spot 100 μ l of the mating mixture in a spiral fashion onto each filter, ensuring the liquid is spread across the filter area. Allow to air dry.
10. Include a donor and recipient control:
 - a. Spot 50-100 μ l of just the *B. abortus* cell culture (recipient control) onto a 25 mm sterile filter on a mating media agar plate. Repeat for *E. coli* cell culture (donor control).
 - b. Two 25 mm filters can be on the same agar plate so long as no cross contamination occurs.
11. When all filters are dry, place the plates in 37°C incubator overnight.

Day 1

1. Filters should have moderate growth after 16-24 hr incubation. Using sterile flamed forceps, fold each filter in half, and transfer to a sterile conical tube, with care not to contaminate the filter in the process.
2. Add 1 ml 2YT without DAP to all tubes.
3. All tubes should be gently vortexed to dislodge the cells, resulting in a turbid cell suspension.
4. Pool all mating suspensions to a single tube, if more than one filter mating performed.
This is the conjugated Tn-Seq Library pool.
 - a. Take a small aliquot of this pool for serial dilutions and plating:
 - i. 200 μ l each of pool, serially diluted ten-fold through 10^{-9}

1. Plate 100 μ l of each dilution on 2YT without DAP, and 2YT without DAP + selection antibiotic
 - a. *No antibiotic: Determines the total available recipient cells after conjugation.*
 - b. *Antibiotic: Determines total number of successful conjugants*
- ii. 100 μ l each of undiluted recipient control, and 100 μ l of undiluted donor control onto 2YT plates without DAP + antibiotic
 1. *Confirms selectivity of 2YT -DAP + antibiotic plates*
 2. *You may also plate donor control onto LB + Dap + antibiotic to calculate transconjugant to donor ratio if desired*
- iii. Spread all plates evenly (e.g. by glass beads or L spreader), allow to dry.
- iv. Incubate in 37°C for 2-4 days.
 1. Check plates daily for growth
- b. Transfer 900 μ l aliquots of the conjugated library pool into microcentrifuge or cryogenic tubes. Add 100 μ l of DMSO to each.
- c. Store aliquots in -80°C until further use.

Day 4-5

1. Count colony forming units (CFUs) on the 2YT + antibiotic plates at the lower dilutions to calculate the concentration of mutants/ml in the pool
 - a. Important to check that there is not also growth on the control plates, which would suggest the presence of antibiotic suppressors

Library Collection

Day 0

1. Thaw the desired number of conjugated library pooled aliquots based on estimated number of mutants/aliquot and needs of the experimental design.

Note: If high mutant density (~1-2 million mutants/ml), dilute each aliquot 1:10 in 2YT + antibiotic media.
2. Plate 500 μ l of thawed aliquot or 1:10 dilution onto a large 150 mm 2YT + antibiotic plate. Spread liquid evenly.
 - a. Repeat until the desired number of mutants plated has been achieved. For example, if the pooled library contains 1.5 million mutants/ml, 1 ml of the 1:10 dilution contains 150,000 mutants that would be collected from 2 large plates.

Note: If density of colonies is a concern, then plate 250 μ l per plate and double the amount of plates to collect from.
3. Place all plates in a 37°C incubator for 2 days.
 - a. Check on the incubating plates daily, remove if ready for collection.

- b. Ideally, the large plates should be pulled for collection when individual colonies are present and densely packed, but a confluent lawn is not yet present.

Day 2

1. For each large plate, add 3 ml of 2YT onto the plate.
2. Take a sterile cell scraper and gently scrape the colonies to a corner to mix/resuspend in the liquid. Avoid disturbing the agar.
 - a. If the scraper blade is full of cells, you can take a sterile pipet tip and scrape the blade and transfer the clump of bacteria to the walls of a sterile culture tube.
3. When most of the cells have been scraped to a corner of the plate, aspirate the remaining liquid and transfer to the culture tube. Sometimes the liquid is absorbed by the agar and you may have to add more liquid to aspirate. When as much of the cells have been collected as possible, vortex the culture tube to mix the very turbid culture together.
4. Repeat this for all plates in the mutant collection. Pool the cultures together in a single sterile tube. Resuspend with 2YT for desired final volume, which depends on the DNA extraction method used. (1 ml may be a sufficient start to freeze initially).
 - a. The pellet can be frozen at -20°C until thawing for experimental outgrowth begins.
5. *Optional:* If enumeration of scraped library pools is desired, take a small aliquot and serially dilute in a 96 well plate to a dilution factor of at least 10^{-10} or 10^{-11} . Either plate 100 μl or spot 10 μl of the lowest 6 dilutions in duplicate onto an 2YT + antibiotic plate. Incubate at 37°C x 2 - 4 days.
6. **Safe Stopping Point.** Make 900 μl aliquots of the scraped pools in microcentrifuge tubes. Based on resuspension volumes, keep track of how many mutants are estimated to be present in each aliquot. For example, if 2 scraped plates representing 150,000 mutants total are resuspended in ~ 2 ml, two 1 ml aliquots represent the set of 150,000 mutants. Add 100 μl DMSO to each tube and freeze at -80°C .

Note: Large scale bacterial culture may encounter challenges with DNA extraction. Scalable protocols such as chloroform:phenol:isoamyl alcohol methods are recommended.

Suggested Thermofisher protocol

<https://www.thermofisher.com/us/en/home/references/protocols/nucleic-acid-purification-and-analysis/dna-extraction-protocols/phenol-chloroform-extraction.html>

References

1. Ferrières L, Hémerly G, Nham T, Guérout A-M, Mazel D, Beloin C, Ghigo J-M. 2010. Silent mischief: Bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *J Bacteriol* 192:6418–6427.
2. Jackson SA, Fellows BJ, Fineran PC. 2020. Complete genome sequences of the *Escherichia coli* donor strains ST18 and MFDpir. *Microbiol Resour Announc* 9:e01014-20.