Transposon Mutagenesis (Mulvey Lab)

Transposon mutagenesis using EcS17 donor strain: Auxotrophies- Thi, Pro

pSAM_Ec derived from pSAM_Bt

Media required:

1. Wash media: 1x M9 salts (no supplements except $MgSO_4$ and $CaCl_2$)
2. Pre-mating media: 1x M9-thr-pro-thi-glucose broth
3. Mating agar: 1x M9-thr-pro-thi-glucose agar
4. Selective agar: 1x M9-thr-glucose-kan agar
5. Selective media: 1x M9-thr-glucose broth (add kanamycin to select for transposon mutants when needed)

[notes on media prep]

- M9 salts: 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$
- add all supplements including nicotinic acid, $CaCl_2$ and $MgSO_4$ to the M9 salts after the autoclaving
- Concentrations:
    - threonine = 40 µg/ml
    - proline = 40 µg/ml
    - thiamine = 1 µg/ml
    - glucose = 0.2% w/v
    - kanamycin = 50 µg/ml

Pre-mating outgrowth:

*Donor- EcS17/pSAM-Ec***:**

Grow in 5 mL pre-mating media, shaking at 37^{o}C in the presence of ampicillin (100 µg/ml) to an optical density at 600 nm (OD_{600}) of ~0.5 to 0.8. Collecting the donor strain after it reaches mid-log growth phase is optimal for mutagenesis. EcS17/pSAM-Ec needs ~36 to 48 h to reach an OD_{600} 1.0. To coordinate with the recipient pre-mating culture, subculture the donor strain (1:25, or 200 µl in to 5 ml) from a ~36 to 48 h culture

into fresh pre-mating media plus ampicillin. It will then take ~7 h to reach the proper OD_{600} of 0.5 to 0.8.

Recipient- ExPEC strain F11:

In our lab we have mutagenized various *E.coli* pathogens and non-pathogens with the pSAM-Ec vector and EcS17, but efficiency varies, and optimization may be required for each strain. Grow the recipient strain in pre-mating media overnight, shaking at 37^{o}C. In the morning, subculture the recipient strain into 5 ml fresh pre-mating media and culture until an OD_{600} of 0.5 to 0.8 is reached. Heat-shock the recipient for 30 minutes at 50^{o}C.

Example protocol chronology:

Day 1:

1. In the morning, start a 5 mL culture of donor strain (EcS17/pSAM-Ec) in pre-mating media plus ampicillin.

Day 2:

1. Start overnight culture of recipient strain in pre-mating media.

Day 3:

1. Subculture donor strain into 5 mL pre-mating media plus ampicillin. Incubate about 7 hours to obtain OD_{600} of 0.5 to 0.8. Subculture the recipient strain so that it reaches a similar OD_{600} at the same time as the donor.

2. Heat shock recipient strain at 50° C for 30 minutes.

3. Measure OD_{600} of both the donor and recipient cultures. Dilute the recipient using M9 salts to an OD_{600} that is half the OD_{600} of the donor. This dilution is used to achieve a recipient cell concentration approximately half that of the donor.

4. Add 750 µl each of donor and recipient culture to a microcentrifuge tube to form the mating mixture (1.5 ml total volume).

5. Wash #1- pellet the mixture by centrifuging at high-speed, aspirate supernatant, resuspend in 500 µl M9 salts.

6. Wash #2- pellet the mixture by centrifuging at high-speed, aspirate supernatant, resuspend in 150 µl pre-mating media.

7. Place a sterile 0.45 µm membrane disc (Millipore, cat. no. HAWP02500) onto the surface of a mating agar plate. Apply 50 µl of the mating mixture to the surface of the membrane. Multiple matings can be performed from one mating mixture.

8. Incubate plates upright at 37^{o}C for 5 hours.

9. Remove individual membranes from mating agar plates with sterile tweezers and place in a 50 mL conical tube containing 2 mL M9 salts. Dislodge bacteria by vortexing.

10. Gently centrifuge to collect all liquid in the bottom of the tube.

11. Serially dilute and plate 100 µl of the mating suspension on selective agar plates and incubate at 37^{o}C overnight. If mutagenesis efficiency is high, may need to plate 10^{-1} and 10^{-2} dilutions. These plates will be used to estimate mutagenesis frequency and infer how many distinct mutants were generated.

12. Add the remainder of the mating suspension to a 250 mL flask containing 20 mL of selective media. Incubate shaking at 37^{o}C for 40 to 60 min to allow transposon mutants time to express the kanamycin resistance gene. After this initial growth period, add the kanamycin antibiotic and continue to culture until an OD_{600} of 0.4 to 0.5 is reached. This normally takes about 2-3 h total.

13. Optional: Serially dilute and plate the mutant outgrowth culture onto Luria Bertani (LB) agar plates +/- kanamycin to estimate purity of the mutant library. We observed our mutant libraries to be nearly 100% pure using this procedure. Additionally, another optional serial dilution and plating can be done using LB agar plates +/- ampicillin to ensure that mutants no longer carry the pSAM-Ec plasmid.

14. Freeze down 1 mL freezer stocks—at least 1 stock for long-term storage/use and 1 axillary stock for thawing and combining with other mutant libraries. Mix 1 mL of culture and 0.5 mL 80% glycerol for storage at -80^{o}C.

Day 4:

1. Count plates containing serial dilutions from the mating suspension (Day 3, step 11). Calculate mutagenesis frequency (transposon hops per mating). This number can be used to infer how many distinct transposon mutants are contained within each stock. NOTE: this calculation is purely an estimate and will

likely differ after determination by deep sequencing. However, we did find this number was reliable for use in the design and execution of our selection screen.

This protocol was assembled and optimized by Travis Wiles with the help of Stephanie Aoki (David Low Lab, UC Santa Barbara) and Colin Russell (Matt Mulvey Lab, University of Utah).

References/Resources:

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