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Title: Generating Transposon Insertion Libraries In Gram-Negative Bacteria For High-Throughput Sequencing

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 18
Number of Shots: 49

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Misha Kazi:** This method is significant because it can be used in diverse bacterial species to identify essential genes, antibiotic resistance genes and genes important for *in vivo* fitness during infection.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Misha Kazi:** The main advantage of this technique is the mechanical shearing of genomic DNA and poly-C tail addition, which eliminate the need for expensive restriction enzymes, adaptor ligation and gel purification.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Richard Schargel:** The implications of this technique extend toward the therapy of problematic bacterial infections because genes identified as important for infection or antibiotic resistance could serve as targets for novel antimicrobial therapeutics.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Richard Schargel:** This method can provide insight into complex genotype-phenotype relationships across Gram-negative and Gram-positive bacterial species and improve our current understanding of bacterial genetics.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Bacterial Mating

2.1. To begin, transfer overnight cultures to 50-milliliter conical tubes **[1]** and pellet both recipient and donor cultures using centrifugation at 5,000 *x g* for 7 minutes **[2]**. Discard the supernatant. **[3]** Use a 10-milliliter serological pipette to resuspend the "donor" strain pellet in 4.5 milliliters of LB supplemented with diamino pimelic acid **[4]**.

2.1.1. WIDE: Establishing shot of talent transferring cultures into tubes.

2.1.2. Talent putting the tube in the centrifuge and closing the lid, with the tubes labeled "donor" or "recipient".

2.1.3. Talent removing the supernatant.

2.1.4. Talent resuspending the pellet.

2.2. Transfer the resuspended "donor" strain into the "recipient" strain tube **[1]** and immediately distribute the mating suspension as individual 100-microliter droplets on LB agar plates supplemented with diamino pimelic acid **[2-TXT]**. Incubate the plates at room temperature for 30 minutes **[3]**.

2.2.1. Talent transferring the donor strain into the recipient strain tube, with both tubes labeled.

2.2.2. Talent distributing droplets on an LB agar plate. **TEXT: 5 - 7 droplets per plate**

2.2.3. Plate incubating at RT.

2.3. Then, carefully transfer the plates to a 37-degree Celsius incubator and allow the cultures to mate for 1 hour **[1]**. After the incubation, add 1.5 milliliters of LB onto each plate **[2]** and harvest the bacteria into a 50-milliliter conical tube **[3]**.

2.3.1. Talent putting the plate in the incubator and closing the door.

2.3.2. Talent adding LB to a plate.

2.3.3. Talent transferring bacteria into a 50mL tube.

2.4. Pellet the mated cells using centrifugation at 5,000 *x g* for 7 minutes **[1]**, then discard the supernatant **[2]** and resuspend the cells in 50 milliliters of LB to remove residual diamino pimelic acid **[3]**. Pellet the cells again and repeat the wash with LB **[4]**.

2.4.1. Talent putting the tube in the centrifuge and closing the lid. *Videographer: Obtain multiple usable takes, this will be reused in 2.4.4.*

2.4.2. Talent removing the supernatant.

2.4.3. Talent resuspending the cells.

2.4.4. *Use 2.4.1.*

2.5. When finished, use a 10-milliliter serological pipette to resuspend the pellet in 10 milliliters of LB supplemented with 25% glycerol **[1]**. Spread the cells on plates according to manuscript directions **[2]**, then incubate the plates at 37 degrees Celsius overnight **[3]**.

2.5.1. Talent resuspending the pellet in media.

2.5.2. Talent spreading bacteria on a plate.

2.5.3. Talent putting the plate in the incubator and closing the door.

2.6. Create 1-milliliter aliquots of the remaining bacteria and store them at -80 degrees Celsius **[1]**.

2.6.1. Talent putting aliquots in the freezer and closing the door.

3. Generation of Final Bacterial Mutant Library

3.1. Thaw an aliquot of the frozen mating on ice **[1]**, then use sterile glass beads to spread 150 microliters of the dilution onto each 30 by 150-millimeter Luria-Bertani agar plate supplemented with kanamycin **[2]**. Dispose of the used tube containing excess mating **[3]** and incubate plates at 37 degrees Celsius for 14 hours **[4]**.

3.1.1. Talent putting an aliquot on ice.

3.1.2. Talent spreading bacteria on a plate.

3.1.3. Talent disposing of the excess mating.

3.1.4. Talent putting the plates in the incubator and closing the door.

3.2. After the incubation, count the colony forming units on each plate to estimate the total mutants in the transposon library **[1]**. Count 20% of at least 3 plates to determine the colony count estimate for the entire group of plates **[2]**.

3.2.1. Talent counting the CFUs.

3.2.2. Close up of the plate with the colonies.

3.3. After calculating the estimated colony yield, add 3 to 5 milliliters of LB to each plate **[1]** and scrape off the bacteria using a sterile scraping tool **[2]**. Pool bacterial suspensions from all plates into 50-milliliter conical tubes **[3]** and pellet the suspensions by centrifuging at 5,000 *x g* for 7 minutes **[4]**. *Videographer: This step is important!*

3.3.1. Talent adding LB to a plate.

3.3.2. Talent scraping off the bacteria.

3.3.3. Talent adding bacterial suspension to a 50mL tube.

3.3.4. Talent putting the tube in the centrifuge and closing the lid.

3.4. Discard the supernatant and resuspend the pellet in 5 milliliters of LB supplemented with 30% glycerol **[1]**, then make 1-milliliter aliquots of the transposon library in cryovials and store them at -80 degrees Celsius **[2]**.

3.4.1. Talent removing the supernatant and adding LB to the tube.

3.4.2. Talent aliquoting the library into cryovials.

4. DNA Shearing and Poly-C Tail Addition

4.1. Dilute the gDNA with TE buffer to a concentration of 250 nanograms per microliter in a total volume of 200 microliters **[1]** and place it in the water bath sonicator **[2]**. Sonicate the DNA to yield fragments of approximately 300 nucleotides **[3-TXT]**. *Videographer: This step is important!*

4.1.1. Talent diluting the gDNA.

4.1.2. Talent placing the gDNA tubes in the sonicator.

4.1.3. Talent programming and starting the sonicator. **TEXT: Power: 60 %; Total Time: 20 minutes; Cycles: 10 seconds ON and 10 seconds OFF; 4 ˚C**

4.2. Confirm that the DNA is sheared by running 10 microliters of unsheared DNA and 10 microliters of sheared DNA on a 1% agarose gel. Repeat the sonication if needed **[1]**.

4.2.1. Gel running. *Video Editor: Show Figure 3 B here as a split screen or inset.*

4.3. To add the poly-C tail to the 3-prime end of the sheared DNA, setup the poly-C reaction as described in the text manuscript **[1]** and incubate the reaction tubes for 1 hour at 37 degrees Celsius **[2]**.

4.3.1. Talent putting reagents in tubes.

4.3.2. Talent putting the tubes in the incubator.

4.4. To purify the poly-C reaction, add 40 microliters of size-selection paramagnetic beads to each sample **[1]** and vortex the reaction tube **[2]**. Incubate samples at room temperature for 5 minutes, then briefly centrifuge them to collect the liquid at the bottom of the tube **[3]**. *Videographer: This step is important!*

4.4.1. Talent adding beads to a sample.

4.4.2. Talent vortexing the tube. *Videographer: Obtain multiple usable takes, this will be reused in 4.7.2.*

4.4.3. Talent centrifuging the tube. *Videographer: Obtain multiple usable takes, this will be reused in 4.6.2 and 4.7.3.*

4.5. Transfer the tubes to a magnetic rack and incubate them at room temperature for 2 minutes or until the solution is clear **[1]**. Carefully remove the supernatant **[2]** and add 200 microliters of freshly prepared 80% ethanol without disturbing the beads **[3]**. *Videographer: This step is important!*

4.5.1. Talent putting the tubes in a magnetic rack.

4.5.2. Talent removing the supernatant.

4.5.3. Talent adding ethanol to a tube, with the ethanol container in the shot.

4.6. Incubate the samples until the solution is clear, then remove the supernatant and repeat the ethanol wash **[1-TXT]**. Briefly centrifuge the tubes **[2]** and put them back in the magnetic rack to remove any remaining liquid. Incubate the samples at room temperature for 2 to 5 minutes to allow them to dry **[3]**.

4.6.1. Talent removing ethanol from the tubes. **TEXT: Incubate for at least 30 seconds**

4.6.2. *Use 4.4.3.*

4.6.3. Talent putting the tubes back on the rack.

4.7. Remove the tubes from the magnetic rack and add 25 microliters of water to each tube **[1]**. Vortex them for approximately 5 seconds or pipette up and down **[2]**, then centrifuge the tubes to collect liquid at the bottom **[3]**. *Videographer: This step is important!*

4.7.1. Talent adding water to a tube.

4.7.2. *Use 4.4.2.*

4.7.3. *Use 4.4.3.*

4.8. Transfer the tubes to the magnetic rack and let them sit for approximately 2 minutes until the solution is clear, then transfer the supernatant to a new tube without disturbing the beads **[1]**. *Videographer: This step is important!*

4.8.1. Talent transferring supernatant from a tube on the magnetic rack to a new tube.

Results

5. Results: Transposon Mutant Library

5.1. This protocol was used to generate a high-density transposon library in *A. baumannii* strain ATCC 17978 through bacterial conjugation using *E. coli* MFD DAP$^-$ *(pronounce 'dap-auxotrph')*, which replicates the plasmid pJNW684 **[1]**.

5.1.1. LAB MEDIA: Figure 4 B.

5.2. The pooled *A. baumannii* transposon mutant library was used to identify fitness factors important for colistin resistance under subinhibitory concentrations of the antimicrobial **[1]**.

5.2.1. LAB MEDIA: Figure 2 B.

5.3. After depleting the mutant library of genes that contribute to colistin resistance, the total gDNA of the remaining library pool was isolated from control and experimental cultures. The gDNA was fragmented with mechanical shearing and a poly-C tail was added to the DNA fragments in preparation for sequencing **[1]**.

5.3.1. LAB MEDIA: Figure 3.

5.4. DNA concentrations were calculated and the samples were analyzed using chip-based capillary electrophoresis to confirm successful library builds **[1]**.

5.4.1. LAB MEDIA: Figure 4 A.

Conclusion

6. Conclusion Interview Statements

6.1. **Richard Schargel:** The most important thing to remember when attempting this procedure is to adjust the mating volume based on CFU counts for specific target strains to generate a high-resolution mutant library. Additionally, the recipient strain must be sensitive to the antibiotic resistance marker used in the transposon prior to mutagenesis.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.2.*

6.2. **Misha Kazi:** Following this procedure, gene deletion or silencing methods can be performed to knockout individual hits obtained from sequencing results and validate genes of interest under challenge conditions.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.