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TITLE:

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

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SUMMARY:

We describe a method to generate saturating transposon mutant libraries in Gram-negative bacteria and subsequent preparation of DNA amplicon libraries for high-throughput sequencing. As an example, we focus on the ESKAPE pathogen, *Acinetobacter baumannii*, but this protocol is amenable to a wide range of Gram-negative organisms.

ABSTRACT:

Transposon sequencing (Tn-seq) is a powerful method that combines transposon mutagenesis and massive parallel sequencing to identify genes and pathways that contribute to bacterial fitness under a wide range of environmental conditions. Tn-seq applications are extensive and have not only enabled examination of genotype-phenotype relationships at an organism level but also at the population, community and systems levels. Gram-negative bacteria are highly associated with antimicrobial resistance phenotypes, which has increased incidents of antibiotic treatment failure. Antimicrobial resistance is defined as bacterial growth in the presence of otherwise lethal antibiotics. The “last-line” antimicrobial colistin is used to treat Gram-negative bacterial infections. However, several Gram-negative pathogens, including *Acinetobacter baumannii* can develop colistin resistance through a range of molecular mechanisms, some of which were characterized using Tn-seq. Furthermore, signal transduction pathways that regulate colistin resistance vary within Gram-negative bacteria. Here we propose an efficient method of transposon mutagenesis in *A. baumannii* that streamlines generation of a saturating transposon insertion library and amplicon library construction by eliminating the need for restriction enzymes, adapter ligation, and gel purification. The methods described herein will enable in-depth analysis of molecular determinants that contribute to *A. baumannii* fitness when challenged with colistin. The protocol is also applicable to other Gram-negative ESKAPE

pathogens, which are primarily associated with drug resistant hospital-acquired infections.

INTRODUCTION:

The discovery of antibiotics is undoubtedly one of the most impactful health-related events of the 20th century. Not only do antibiotics quickly resolve serious bacterial infections, they also play a pivotal role in modern medicine. Major surgeries, transplants and advances in neonatal medicine and chemotherapy leave patients susceptible to life threatening infections and these therapies would not be possible without antibiotics[1,2]. However, rapid development and spread of antibiotic resistance among human pathogens has significantly decreased the efficacy of all clinically important classes of antibiotics[3]. Many bacterial infections that were once easily cleared with antibiotics treatment, no longer respond to classic treatment protocols, causing a serious threat to global public health[1]. Antimicrobial resistance (AMR) is where bacterial cells grow in otherwise lethal concentrations of antibiotics, regardless of the treatment duration[4,5]. There is an urgent need to understand molecular and biochemical factors that regulate AMR, which will help guide alternative antimicrobial development. Specifically, ESKAPE pathogens are problematic in clinical settings and associated with extensive AMR. These include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. While several mechanisms contribute to AMR in ESKAPE pathogens, the latter four organisms are Gram-negative.

Gram-negative bacteria assemble a defining outer membrane that protects them from adverse environmental conditions. The outer membrane serves as a permeability barrier to restrict entry of toxic molecules, such as antibiotics, into the cell. Unlike other biological membranes, the outer membrane is asymmetrical. The outer leaflet is enriched with surface-exposed lipopolysaccharide, while the inner leaflet is a mixture of phospholipids[6]. Lipopolysaccharide molecules are anchored to the outer membrane by a conserved lipid A moiety embedded within the lipid bilayer[7]. The canonical lipid A domain of *Escherichia coli* lipopolysaccharide is required for the growth of most Gram-negative bacteria and is synthesized by a nine-step enzymatic pathway that is one of the most fundamental and conserved pathways in Gram-negative organisms[6–8].

Polymyxins are cationic antimicrobial peptides that target the lipid A domain of lipopolysaccharide to perturb the outer membrane and lyse the cell. The electrostatic interaction between positively charged residues of polymyxins and the negatively charged lipid A phosphate groups disrupt the bacterial cell membrane ultimately leading to cell death[9–13]. Colistin (polymyxin E) is a last-resort antimicrobial used to treat infections caused by multidrug resistant Gram-negative nosocomial pathogens, such as *Acinetobacter baumannii*[14–16]. First discovered in 1947, polymyxins are produced by the soil bacteria, *Paenibacillus polymyxa*[17–19]. Polymyxins were prescribed to treat Gram-negative infections for years before their clinical use was limited due to reports of significant nephro- and neurotoxicity[20,21].

A. baumannii is a nosocomial Gram-negative pathogen that has dramatically increased the morbidity and mortality of patient outcomes over recent decades[22]. What was once regarded as a low-threat pathogen, now poses a significant risk for hospital-acquired infection throughout

the world due to its incredible ability to acquire AMR and high risk of epidemic[23,24]. *A. baumannii* accounts for more than 10% of nosocomial infections in the United States. Disease manifests as pneumonia, bacteremia, urinary tract infections, skin and soft tissue infections, meningitis, and endocarditis[25]. Treatment options for *A. baumannii* infections have dwindled due to resistance against almost all antibiotic classes, including β-lactams, fluoroquinolones, tetracycline, and aminoglycosides[23,24]. The prevalence of multidrug resistant, extensively drug-resistant and pan-drug resistant *A. baumannii* isolates has led to a resurgence in colistin treatment, which was thought to be one of the few remaining therapeutic options still effective against multidrug resistant *A. baumannii*. However, increased colistin resistance among *A. baumannii* isolates has further amplified its threat to the global public health[10–13,27,30,31].

Recent advances in high-throughput sequencing technologies, such as transposon sequencing (Tn-seq), have provided important tools to advance our understanding of bacterial fitness in vitro and in vivo. Tn-seq is a powerful tool that can be leveraged to study genotype-phenotype interactions in bacteria. Tn-seq is broadly applicable across bacterial pathogens, where it combines traditional transposon mutagenesis with massive parallel sequencing to rapidly map insertion sites, which can be used to link DNA mutations to phenotypic variants on a genome-wide scale[32–35]. While transposon mutagenesis methods have been previously described, the general steps are similar[33]. First, an insertion library is generated using transposon mutagenesis, where each bacterial cell within a population is restricted to a single transposon insertion within the genomic DNA (gDNA). Following mutagenesis, individual mutants are pooled. gDNA is extracted from the insertion mutant pool and the transposon junctions are amplified and subjected to high-throughput sequencing. The reads represent insertion sites, which can be mapped to the genome. Transposon insertions that reduce fitness quickly fall out of the population, while beneficial insertions are enriched. Tn-seq has been instrumental to advance our understanding of how genes impact bacterial fitness in stress[33].

The *Himar1 mariner* transposon system encoded in pJNW684 was specifically constructed and optimized for the purpose of transposon mutagenesis. It includes a *mariner*-family transposon flanking the kanamycin resistance gene, which is used for the selection of transposon insertion mutants in *A. baumannii.* It also encodes an *A. baumannii* specific promoter that drives expression of the transposase encoding gene[36]. The *mariner*-based transposon also contains two translational terminators downstream of the kanamycin resistance gene, which prevents read-through downstream of the insertion[37]. pJNW684 also carries a RP4/oriT/oriR6K-conditional origin of replication which requires the *λpir* gene contributed by the donor strain to replicate[38]. In absence of the *λpir* gene, the pJNW684 vector carrying the transposition machinery will not be able to replicate in the *A. baumannii* recipient strain[10,36,38]. Therefore, during bacterial conjugation, only the transposon is inserted into the recipient genome without background insertion of the plasmid, which carries the transposase gene. This is significant because the loss of transposase activity along with the plasmid results in single, stable transposition event that prevents the transposon from moving to different locations once it inserts into the recipient genome.

pJNW648 has also been tested for activity in another Gram-negative organism, *E. coli*. Successful

assembly of a saturating Tn-seq library in *E. coli* strain W3110 indicated the system is amenable to perform mutagenesis in a wide range of pathogens, including Enterobacteriaceae. Furthermore, the *A. baumannii* specific promoter that drives transposase expression can quickly be exchanged with a species-specific promoter. Lastly, the kanamycin resistance gene can be exchanged for other resistance cassettes, depending on the AMR phenotype of the organism being studied.

One factor that contributes to colistin resistance in *A. baumannii* is administration of insufficient doses, where bacteria are exposed to selective pressure at non-lethal levels[39]. Several reports showed that subinhibitory antimicrobial concentrations can induce regulated responses that alter cell physiology to reduce susceptibility of the entire bacterial population[11,12,30,31]. Using Tn-seq, we discovered factors that regulate colistin resistance in *A. baumannii* strain ATCC 17978 after exposure to inhibitory[10] and subinhibitory concentrations of colistin. This example details a Tn-seq method that streamlines the construction and enrichment of a saturated transposon mutant library using the *mariner*-based family of transposons[40,41]. While several Tn-seq protocols generate 20,000 - 100,000 mutants[35,42–46], the protocol described herein can rapidly generate a transposon library of 400,000 + mutants, which roughly equates to a transposon insertion every 10-base pairs in *A. baumannii*[10]. Furthermore, the library size can be scaled up without significant additional effort. This method also eliminates the requirement for restriction endonucleases, adapter ligation and gel purification, which can reduce final library diversity.

PROTOCOL:

1. Bacterial strain preparation

1.1 Streak the "donor" strain (*E. coli* MFD DAP$^-$/pJNW684, **Table of Materials**) for isolated colonies on Luria-Bertani agar supplemented with 600 μM diaminopimelic acid (DAP), 100 mg/L of ampicillin and 25 mg/L of kanamycin. Incubate overnight at 37 ˚C. Using a single isolated colony, inoculate 50 mL of Luria broth (LB) supplemented with 600 μM DAP, 100 mg/L of ampicillin and 25 mg/L of kanamycin in a 250 mL Erlenmeyer flask and label it as "donor".

1.2 Streak the "recipient" strain (*A. baumannii* strain ATCC 17978, **Table of Materials**) for isolated colonies on Luria-Bertani agar. Incubate overnight at 37 ˚C. Using a single isolated colony, inoculate 50 mL of LB in a 250 mL Erlenmeyer flask and label it as "recipient".

1.3 Incubate both cultures ("donor" and "recipient") overnight at 37 ˚C with shaking.

2. Bacterial mating

2.1 Transfer overnight cultures to 50 mL conical tubes.

2.2 Pellet both recipient and donor cultures using centrifugation at 5,000 *x g* for 7 min.

2.3 Discard the supernatant and resuspend the “donor” strain pellet in 35 mL of LB supplemented with DAP to wash away residual antibiotics.

2.4 Pellet the “donor” strain cells using centrifugation at 5,000 *x g* for 7 min.

2.5 Discard the supernatant and resuspend the “donor” strain pellet in 4.5 mL of LB supplemented with DAP. Use a 10 mL serological pipette.

2.6 Transfer the resuspended “donor” strain into “recipient” strain tube, which contains the pelleted “recipient” cells. Use the same 10 mL serological pipette from step 2.5 to mix the cultures. Immediately move to the next step.

NOTE: Mating is done at a 10:1 donor:recipient ratio. The total volume of the final suspension should be 5 mL.

2.7 Distribute the mating suspension as individual 100 µL droplets on LB agar plates supplemented with DAP (5-7 droplets per plate) (**Figure 1A**).

NOTE: ~10 plates are required for 5 droplets per plate. ~ 8 plates are required for 7 droplets per plate.

2.8 Incubate plates at room temperature for 30 min.

2.9 Without disturbing the droplets, carefully transfer plates to a 37 ˚C incubator and allow cultures to mate for 1 h**.**

NOTE: Incubation periods exceeding 1 h risks generation of sister mutants.

2.10 Following incubation, add 1.5 mL of LB onto each plate and harvest by resuspending bacteria from the plates. Use a 1 mL micropipette for resuspension. Final volume should be approximately 12 - 15 mL.

2.11 Combine harvested cells in a 50 mL conical tube.

2.12 Pellet the mated cells using centrifugation at 5,000 *x g* for 7 min.

2.13 Discard the supernatant and resuspend cells in 50 mL of LB to remove residual DAP.

2.14 Pellet the mating using centrifugation at 5,000 *x g* for 7 min.

2.15 Repeat the wash step (steps 2.13 and 2.14).

2.16 Using a 10 mL serological pipette, resuspend the pellet in 10 mL of LB supplemented with 25% glycerol.

2.17 Using washed cells, make five serial dilutions in LB broth (1:10, 1:100, 1:1000, 1:10,000, 1:100,000).

2.18 Spread 100 µL of each dilution on 4 different plates using sterile glass beads: Luria-Bertani agar supplemented with kanamycin, agar supplemented with ampicillin, agar supplemented with DAP and agar only.

2.19 Incubate plates at 37 ˚C overnight.

2.20 Aliquot the remaining mating in 1 mL aliquots and store at -80 ˚C.

3. Determine the appropriate dilution of transposon library

3.1 Record colony-forming units (CFU) from overnight plates.

3.2 Image a plate with countable colonies for each different plate condition (**Figure 2A**).

NOTE: Both "donor" and "recipient" strains should grow on agar plates supplemented with DAP, so most plated dilutions will yield a lawn. Only the "recipient" strain can grow on agar plates. Neither the "donor" nor the "recipient" strain can grow on agar plates supplemented with ampicillin, so there should be none/minimal growth. Only target strain cells encoding the transposon insertion can grow on agar plates supplemented with kanamycin. Colonies should vary in size, indicating transposon insertions in genes that contribute to fitness on agar supplemented with kanamycin.

3.3 Calculate the number of transposon mutants in the frozen mating by counting the number of colonies on LB agar plates supplemented with kanamycin.

NOTE: For the *A. baumannii* genome (approximately 4 Mbps), the goal was to obtain about 400,000 colonies in order to generate a high-resolution mutant library (approximately one transposon insertion/10 base pairs). However, this number should be optimized based on genome size of the target species).

4. Generation of final bacterial mutant library

4.1 Thaw an aliquot of the frozen mating on ice.

4.2 Plate mating on 150 mm Luria-Bertani agar plates supplemented with kanamycin based on CFUs calculated in step 3.1. Adjust the volume with LB to yield 13,333 colonies per 150 µL before plating.

NOTE: The CFU count here was determined to be about 10^5 CFU/mL, so the mating volume was adjusted to obtain 13,333 colonies per plate as this would provide an optimal number of colonies on 30 plates for a high-resolution mutant library without overcrowding the plates.

4.3 Use sterile glass beads to spread 150 µL of the dilution per plate on 30 x 150 mm Luria-Bertani agar plates supplemented with kanamycin to obtain 400,000 colonies (**Figure 1C**).

NOTE: Sterile rods or any kind of sterile spreader tool (i.e., glass beads) may be used to spread the bacteria on plates.

4.4 Dispose of the used tube containing excess mating.

NOTE: Freeze/thaw cycles adds selective pressures on the bacterial culture, which can skew the Tn-seq experiment results. Use a fresh aliquot each time.

4.5 Incubate plates at 37 ˚C for 14 h.

NOTE: The incubation time is optimized to prevent overgrowth (colonies touching). Minimizing growth by reducing the incubation time is suggested.

5. Estimating library density and pooling for storage

5.1 Count CFUs on each plate to estimate the total mutants in the transposon library. Count 20% of at least 3 plates to determine the colony count estimate for the entire group of plates (**Figure 2A**). Ensure that the colonies are not touching another colony.

5.2 After calculating the estimated colony yield, add 3-5 mL of LB (or more if needed) to each plate and scrape off the bacteria using a sterile scraping tool.

NOTE: Sterile inoculating loops were used to efficiently scrape plates.

5.3 Pool bacterial suspensions from all plates into 50 mL conical tubes (**Figure 1D**). This will require multiple 50 mL conical tubes, at least 3.

5.4 Pellet pooled bacterial suspension using centrifugation at 5,000 *x g* for 7 min.

5.5 Discard the supernatant and resuspend pellet in 5 mL of LB supplemented with 30% glycerol.

5.6 Aliquot 1 mL of the transposon library into cryovials and store at -80 ˚C.

6. Identification of factors that regulate colistin resistance in *A. baumannii*

6.1 Prepare 4 x 250 mL Erlenmeyer flasks with each containing 50 mL LB broth and label as (**Figure 2B**)

6.1.1 *A. baumannii* strain ATCC 17978 Tn-seq library; (-) colistin_1

6.1.2 *A. baumannii* strain ATCC 17978 Tn-seq library; (-) colistin_2

6.1.3 *A. baumannii* strain ATCC 17978 Tn-seq library; (+) colistin_1

6.1.4 *A. baumannii* strain ATCC 17978 Tn-seq library; (+) colistin_2

NOTE: In the challenge growth described here, each condition, (-) colistin control and (+) colistin challenge, is being tested in duplicate. Therefore, the setup requires 4 x 250 mL Erlenmeyer flasks, two per condition.

6.2 Add 50 µL of 0.5 mg/L colistin to (+) colistin flasks (6.1.3 and 6.1.4) and 50 µL water to (-) colistin flasks (6.1.1 and 6.1.2).

6.3 Thaw a frozen Tn-seq library aliquot from step 5 on ice.

6.4 Pipette 1 µL of the thawed library into 1 mL of PBS.

6.5 Measure the optical density at 600 nm (OD_{600}) and multiply by 1,000.

NOTE: This determines OD_{600} of 1 µL of the Tn-library.

6.6 Based on the calculation in step 6.5, inoculate each flask containing 50 mL LB to a final OD_{600} 0.001.

6.7 Grow cultures in a shaking incubator at 37 ˚C to OD_{600} 0.5.

NOTE: It is important that cultures remain in logarithmic growth phase, so if your strain is at a different OD_{600} during exponential growth, the OD_{600} needs to be adjusted to ensure the culture replicates as many times as possible within logarithmic growth phase. If the culture only replicates 3 times, the power to detect fitness defects in mutants is capped at 3-fold differences, in theory. Since different bacteria have different doubling times, it is important to seed the cultures at a fixed OD_{600} to normalize the starting inoculum. This ensures consistent representation of the entire library in all cultures.

6.8 Harvest cultures using centrifugation at 5,000 *x g* at 4 ˚C for 7 min.

6.9 Remove the supernatant and wash with 50 mL of PBS.

6.10 Pellet using centrifugation at 5,000 *x g* at 4 ˚C for 7 min.

6.11 Remove the supernatant and resuspend the pellet in 1 mL of PBS. Aliquot ~ 200 µL into 5 microcentrifuge tubes.

6.12 Pellet using centrifugation at 5,000 *x g* at 4 ˚C for 5 min. Remove all of the supernatant using a pipette tip.

6.13 Store pellets at -20 ˚C or proceed with gDNA extraction.

7. gDNA extraction

7.1 Thaw one cell pellet on ice.

7.2 Add 0.6 mL lysis buffer (**Table of Materials**) and vortex to completely resuspend the pellet.

7.3 Incubate at 37 ˚C for 1 h.

7.4 Add 0.6 mL of phenol/chloroform/isoamyl alcohol to the sample and vortex vigorously.

7.5 Separate phases using centrifugation in a microcentrifuge at max speed at room temp for 5 min.

7.6 Transfer the upper aqueous phase to a new tube. Avoid disturbing the phase interface during transfer.

7.7 Add an equal volume of chloroform to the aqueous phase obtained above and vortex vigorously.

7.8 Separate phases using centrifugation in microcentrifuge at max speed at room temp for 5 min.

7.9 Transfer upper aqueous phase to a new tube.

NOTE: Be sure to avoid disturbing the interface during transfer.

7.10 Add 2.5x aqueous phase volume of cold 100 % ethanol and mix gently. Precipited DNA will be visible.

7.11 Place tube at -80 ˚C for at least 1 h.

7.12 Pellet DNA using centrifugation at max speed at 4 ˚C for 30 min.

7.13 Carefully remove supernatant without disturbing the DNA pellet and wash with 150 µL of 70 % ethanol by pipetting.

7.14 Pellet DNA using centrifugation at max speed at 4 ˚C for 2 min.

7.15 Carefully remove the supernatant.

7.16 Repeat step 7.14 once. Carefully remove all remaining ethanol.

7.17 Dry DNA by incubating at room temp for 5-10 min.

7.18 Resuspend DNA pellet in 100 µL TE buffer by pipetting.

8. DNA shearing (Figure 3A)

8.1 Dilute gDNA with TE buffer to a concentration of 250 ng/µL in a total volume of 200 µL.

8.2 Place tubes in a water bath sonicator.

8.3 Sonicate DNA to yield fragments of approximately 300 nucleotides. Power: 60 %, Total Time: 20 min, Cycles: 10 s ON and 10 s OFF at 4 ˚C (**Figure 3A**).

8.4 Confirm DNA is sheared appropriately by separating 10 µL of unsheared DNA and 10 µL of sheared DNA on a 1% agarose gel. Repeat sonication or optimize as needed (**Figure 3B**).

9. Poly-C tail addition to the 3' end (Figure 3A)

9.1 Setup poly-C reaction according to the **Table 1.**

9.2 Incubate reaction at 37 ˚C for 1 h.

9.3 Purify poly-C reaction with 40 µL of size-selection paramagnetic beads (**Figure 3A**) by following the steps below.

9.3.1 Add 40 µL of size-selection paramagnetic beads to each sample. Vortex ~ 5 s or pipette up and down.

9.3.2 Incubate samples at room temperature for 5 min.

9.3.3 Briefly, centrifuge tubes to collect liquid in the bottom of the tube (~ 2 s).

9.3.4 Transfer tubes to a magnetic rack and incubate at room temperature for ~ 2 min until the solution is clear.

9.3.5 With tubes on magnetic rack, carefully remove the supernatant.

9.3.6 With tubes on magnetic rack, add 200 µL of freshly prepared 80% ethanol (do not disturb beads).

9.3.7 Incubate samples for at least 30 s until solution is clear.

9.3.8 With tubes on magnetic rack, carefully remove the supernatant.

9.3.9 Repeat the wash step (steps 9.3.6 - 9.3.8).

9.3.10 Collect liquid in the bottom of the tube using a brief centrifugation step (~ 2 s).

9.3.11 Transfer tubes to the magnetic rack and remove any remaining liquid.

9.3.12 Incubate at room temperature for 2-5 min to dry samples. Do not over dry.

9.3.13 Remove tubes from magnetic rack and add 25 µL of water to each. Vortex for ~ 5 s or pipette up and down.

9.3.14 Briefly, centrifuge tubes to collect liquid in the bottom of the tube (~ 2 s).

9.3.15 Transfer tubes to the magnetic rack and allow to sit for ~ 2 min until the solution is clear.

9.3.16 With tubes on the magnetic rack, remove liquid without disturbing the beads and transfer to a new tube (~ 23 µL of DNA).

10. Transposon junction amplification (Figure 3A)

10.1 Setup PCR 1 as described in **Table 1** for the first nested PCR (**Table 2**).

10.2 Perform PCR 1 using conditions described in **Table 1.**

10.3 Purify PCR products with 40 µL of size-selection paramagnetic beads (steps 9.3.1 – 9.3-12). Elute in 50 µL of water (steps 9.3.13 – 9.3.16). At this point the samples can be stored at -20 ˚C.

10.4 Prepare Streptavidin-coupled paramagnetic beads:

10.4.1 Resuspend Streptavidin beads by shaking vigorously.

10.4.2 Add 32 µL of beads per sample to a fresh microcentrifuge tube.

NOTE: Beads for 6 + samples can be prepared in a single tube.

10.4.3 Transfer the tube to a magnetic rack. Incubate for ~ 2 min until solution is clear.

10.4.4 With tube on magnetic rack, remove supernatant.

10.4.5 Remove the tube from magnetic rack. Wash beads by resuspending in 1 mL 1x buffer by pipetting up and down.

10.4.6 Transfer the tube to magnetic rack. Incubate for ~ 2 min until solution is clear.

10.4.7 With the tube on magnetic rack, remove the supernatant.

10.4.8 Repeat wash step with 1 mL 1x (steps 10.4.5 – 10.4.7) twice more for a total of 3 washes.

10.4.9 Remove tube from magnetic rack and resuspend beads in 52 μL of 2x buffer per sample.

10.5 Combine 50 μL of the prepared beads with 50 μL of purified PCR1 (from step 10.3). Pipette to mix.

10.6 Rotate at room temperature for 30 min.

NOTE: The beads can be rotated at room temperature for up to 1 h.

10.7 Wash away unbound DNA:

10.7.1 Briefly, centrifuge to collect liquid at the bottom of the tube (~ 2 s).

10.7.2 Transfer the tube to magnetic rack. Incubate for ~ 2 min until the solution is clear.

10.7.3 With the tube on magnetic rack, remove the supernatant.

10.7.4 Remove the tube from magnetic rack, wash beads by resuspending in 100 μL 1x B&W buffer and pipetting up and down.

10.7.5 Transfer tube to magnetic rack. Incubate for ~ 2 min until the solution is clear.

10.7.6 With tube on magnetic rack, remove supernatant.

10.7.7 Repeat wash steps with 100 μL LoTE (steps 10.7.4 – 10.7.6) twice more for a total of 3 washes.

10.8 Setup PCR 2 as described in **Table 1** to amplify transposon junctions and add a single barcode to each sample (**Table 2** and **Table 3**).

10.9 Perform PCR 2 using conditions described in **Table 1**.

10.10 Purify with 40 μL of size-selection paramagnetic beads (steps 9.3.1 – 9.3.12). Elute in 17 μL of water (steps 9.3.13 – 9.3.16), collect ~ 15 μL.

10.11 Quantify DNA concentration using a fluorometer. The final concentrations should be ~ 50 to 250 ng/μl.

10.12 Assess DNA quality using chip-based capillary electrophoresis (**Figure 4A).**

REPRESENTATIVE RESULTS:

The outlined methods describe the generation of a high-density transposon library in *A. baumannii* strain ATCC 17978 through bacterial conjugation using *E. coli* MFD DAP$^-$, which replicates the plasmid pJNW684 (**Figure 4B**). The detailed protocol uses bi-parental bacterial conjugation for transfer of pJNW684 from the *E. coli λpir*+ donor strain to the *A. baumannii* recipient strain. This is an efficient and inexpensive method for generating dense transposon mutant libraries. Bacteria were mixed at optimized ratios and matings were spotted on Luria-Bertani agar plates for 1 h (**Figure 1A**). During mating, the transposon was transferred from the donor to recipient strain, where it inserted into the gDNA (**Figure 1B**). Matings were collected, approximately 10^5 CFU/mL were calculated and plated on 150 mm x 15 mm agar plates supplemented with kanamycin**.** After 14 h of growth at 37 °C, plates contained thousands of colonies of varying size (**Figure 1C**) indicating successful generation of a transposon mutant library. The transposon insertion mutants were pooled (**Figure 1D**) and frozen in aliquots to prevent repeated freeze-thaw cycles, which could add selective pressure on the insertion library. The pooled *A. baumannii* transposon mutant library was used to identify fitness factors important for colistin resistance under subinhibitory concentrations of the antimicrobial (**Figure 2B**). The mutant library was grown to logarithmic phase in the absence/presence of 0.5 mg/L colistin in duplicate to deplete mutant cells encoding insertions in genes that contribute to colistin resistance. The total gDNA of the remaining library pool was isolated from control and experimental cultures and processed to prepare DNA for sequencing (**Figure 3A**).

Isolated gDNA was fragmented using mechanical shearing and a poly-C tail was added on the DNA fragments (**Figure 3A**). Following the addition of poly-C tail, DNA was purified and the transposon-genome junctions were enriched using the poly-C specific primer followed by a second round of PCR using another poly-C specific primer that introduced the P7 sequencing site which is required for binding the Illumina flow cell and cluster generation, and a six-base barcode that is used to demultiplex libraries post sequencing[37,47]. DNA concentrations were calculated and the samples were analyzed using chip-based capillary electrophoresis to confirm successful library builds (**Figure 4A**), which are ready for high-throughput sequencing.

DNA libraries were sequenced by next generation sequencing. A single-end, 50 cycle run was performed, which yielded 30 million high-quality reads/sample providing 62.5-fold coverage of the transposon library. Transposon junctions (reads) were mapped to the reference genome[48], using commercially available bioinformatics analysis software. The number of reads at each insertion site in the input samples, (-) colistin control condition, were compared to the number of reads in the output samples, (+) colistin experimental condition, and fitness scores for each

insertion site were calculated. The fitness scores were then grouped by gene. Genes demonstrating reduced scores when the library was grown in the presence of colistin relative to the input samples were considered fitness determinants for *A. baumannii* survival at subinhibitory concentrations of colistin. For example, transposon insertions within the PmrAB two-component system were present in the input sample, but were not found in the output sample. PmrAB directly regulates expression of *pmrC*, which transfers phosphoethanolamine onto lipid A to neutralize the charge on the cell surface[12,31]. Neutralization of bacterial surface charge is thought to reduce the electrostatic potential required for colistin-mediated killing. Identification of depleted genes known to contribute to the resistance phenotype validated the method.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of transposon mutant library construction. (**A**) Bacterial conjugation. The "donor" strain, which encodes the transposition machinery, was mixed with the "recipient" strain. The mixture was spotted on LB agar plates and allowed to mate for 1 h. (**B**) Generation of transposon library. The plasmid carrying the transposition machinery was transferred from the "donor" strain to the "recipient" strain and the transposon was randomly inserted throughout the genome of the "recipient" strain. (**C**) Selection. Resulting cells were plated on agar plates supplemented with kanamycin to select for transposon insertion mutants. (**D**) Pooled library. Colonies were scraped from plates, resuspended in LB and pooled.

Figure 2: Representative bacterial conjugation results and a schematic of the colistin Tn-seq experiment. (**A**) Representative kanamycin selection plate. The plate is divided into five equal sections. Blue dots represent colony counting for estimation of *A. baumannii* transposon insertion mutants. At least three separate plates were counted to calculate the final estimation. (**B**) Identification of fitness factors at subinhibitory colistin concentrations. The pooled transposon library was grown to logarithmic growth phase either in the absence (control) or in the presence (experimental) of colistin. Once the cultures reached appropriate optical density, the cells were pelleted and gDNA was extracted from each sample. Each condition was tested in duplicate for a total of four samples.

Figure 3: Flowchart of the DNA amplicon library build for massive parallel sequencing and representative sheared gDNA. (**A**) Tn-seq DNA library build schematic. Following extraction, gDNA was fragmented via mechanical shearing. Terminal deoxynucleotidyl transferase was used to add a poly-C tail to the 3' end of fragmented DNA before PCR amplification of the transposon-genome junctions and barcode addition. (**B**) 1% agarose gel of unsheared and sheared *A. baumannii* mutant libraries following gDNA shearing step. 1 Kb ladder was used as a DNA marker. Sheared gDNA smear primarily ranges between ~ 100 and 500 base pairs.

Figure 4: Representative quality control (QC) results and map of the plasmid carrying the transposition genes. (**A**) QC trace for a DNA library build. There is a peak at ~ 350 base pairs, indicating successful library build. If some larger DNA was detected in the QC results, the samples can be cleaned up further to remove large DNA fragments. (**B**) Plasmid pJNW684 consists of a *Himar1 mariner* transposon (green) with a kanamycin resistance cassette (purple) for mutant

selection, a gene encoding the hyperactive *mariner Himar1* C9 transposase (red) under control of an *A. baumannii* 17978 specific promoter (blue), an ampicillin resistance gene (*bla*, orange) and a RP4/oriT/oriR6K-conditional origin of replication (yellow).

Table 1: Reaction setup. Setup and conditions for poly-C, PCR 1 and PCR 2 reactions.

Table 2: PCR amplification primers. PCR amplification primers used in the protocol to amplify the transposon junctions. The purpose of each is listed in the first column.

Table 3: Barcode primers. Barcode primers are used in the second PCR step to amplify the transposon junctions while adding a P7 sequencing site and barcodes to the amplicon. Not all barcode primers are needed to generate a library. Only barcode primers for the number of samples are required.

DISCUSSION:

A. baumannii is an emerging threat to global public health due to the rapid acquisition of AMR against "last-line" therapeutics, such as colistin[10–12,23,24,30,31]. In recent decades, Tn-seq has played a critical role in elucidating genotype-phenotype interactions across numerous bacterial species and expanding our understanding of bacterial genetics[34,35,42,43]. Tn-seq protocols have been instrumental in identifying essential genes in diverse bacterial species such as *Campylobacter jejuni, Staphylococcus aureus,* the periodontal pathogen *Porphyromonas gingivalis*, and even *Mycobacterium tuberculosis*[37,49–51]*.* Beyond identification of essential genes, Tn-seq has been used to identify antibiotic resistance genes in *Pseudomonas aeruginosa*, several conditionally essential genes in *Salmonella typhimurium,* and numerous genotype-phenotype relationships in *Streptococcus pneumoniae*[52–54]. More recently, transposon sequencing of *Vibrio cholerae* was employed in the infant rabbit model of Cholera to identify genes that are important for in vivo fitness during infection[47]. These studies demonstrate the versatility of Tn-seq as it can be utilized for both in vitro and in vivo studies.

The main advantage of Tn-seq over other methods, such as microarray technologies, 2D gel electrophoresis, and qPCR, is that it does not require prior knowledge of the genome or genomic information[55]. Therefore, transposon mutagenesis coupled with massive-parallel sequencing enables the study of known genes and genomes as well as discovery of novel genetic interactions. Here we have presented a comprehensive method for generating a highly dense transposon mutant library in *A. baumannii* to identify factors that are essential for bacterial fitness when exposed to subinhibitory concentrations of colistin. The described method has also been successfully used in *E. coli* (unpublished data), demonstrating the system is amenable to perform Tn-seq analysis in other Gram-negative pathogens, including Enterobacteriaceae.

Using *mariner* transposons for insertional mutagenesis has several advantages. The transposon family originated from eukaryotic hosts and have been widely used to generate saturating mutant libraries in diverse bacterial populations. *Mariner* transposons are host-independent, which means that stable random insertions can be achieved in the absence of specific host factors[40,41]. Additionally, *mariner* transposons have a defined number of insertion events because

they preferentially insert into thymine-adenine ("TA") motifs, which reduces insertional bias and leads to more robust statistical analysis[37,56–58].

Several *mariner*-based Tn-seq methods use *MmeI* restriction digestion for gDNA fragmentation[32,42,43]. While enzymatic DNA fragmentation is a popular and successful method, it adds unnecessary steps to the procedure and increases potential bias[37]. Not only do these techniques require large quantities of starting materials, they can also potentially lead to unequal representation of insertion sequences in downstream analyses[37,59]. Like some other methods that do not rely on *MmeI* nuclease activity[52,60,61], the method outlined herein relies on mechanical shearing to fragment gDNA, and TdT to add a poly-C tail to the 3' end of the DNA fragments. Compared to enzymatic DNA fragmentation and adapter ligation methods, this approach requires significantly smaller amounts of starting DNA while providing more consistent results, it also lowers the risk of DNA cross-contamination and reduces sample loss due to confinement in a sealed tube[37,59,62]. Furthermore, this method yields longer, high quality sequencing reads of 50 nucleotides which aid in more effective and precise mapping of sequences and a more robust downstream analysis[37,59]. The addition of a synthetic poly-C tail disregards potential overhangs that may result from fragmentation as this method relies on the exogenously added poly-C tail as a recognition site for the reverse primer, which contains 16 dG nucleotides at its 3' end and a sequence specific to next generation sequencing (e.g., Illumina sequencing) at the 5' end, to prime synthesis[47,59]. The use of a synthetic nucleotide tail expands the application of this method to many distinct genomes independent of their native content[59]. Subsequently, transposon-genome junctions are amplified using the poly-C specific primer[37]. This alternative simplifies the procedure by eliminating the need for expensive restriction enzymes, adapter ligation, formation of adapter dimers and gel purification steps. We have further optimized the protocol to efficiently generate highly saturated transposon insertion libraries in several Gram-negative ESKAPE pathogens, including *Acinetobacter baumannii* and can be used to study genetic interactions under diverse *in vitro* and *in vivo* conditions[10].

One limitation of Tn mutagenesis is it relies on the antibiotic resistance markers for selection. However, many Gram-negative ESKAPE pathogens are multidrug or extensively drug resistant, meaning the user may need to exchange the resistance cassette according to the specific pathogen of interest. Furthermore, some clinical isolates are not amenable to transposon mutagenesis using the mariner-based transposon.

A critical step of the protocol is calculating the number of Tn mutants to plate. Plating too many colonies will result in a lawn that can complicate downstream analysis. If the colonies are too close or touching, they can add unwanted selective pressure on the library that can result in artifacts. Ideally, colonies would be not be touching and spaced evenly across the plate, as demonstrated (**Figure 2A**). Conversely, if too few colonies are plated, it will be difficult to isolate multiple Tn insertions in each gene.

It is also important to perform the controls listed in step 2.18. As stated in the Note of section of 3. 2, neither "donor" or "recipient" strain should grow on plates supplemented with Ampicillin. Since exogenous DAP is required for growth of the "donor" strain, any growth would indicate the

"recipient" strain replicates pJNW684. This is a significant problem because if the transposon does not integrate into the gDNA, sequencing reads will only map to the plasmid, not an integration site. In this case the experiment will likely not yield useable data.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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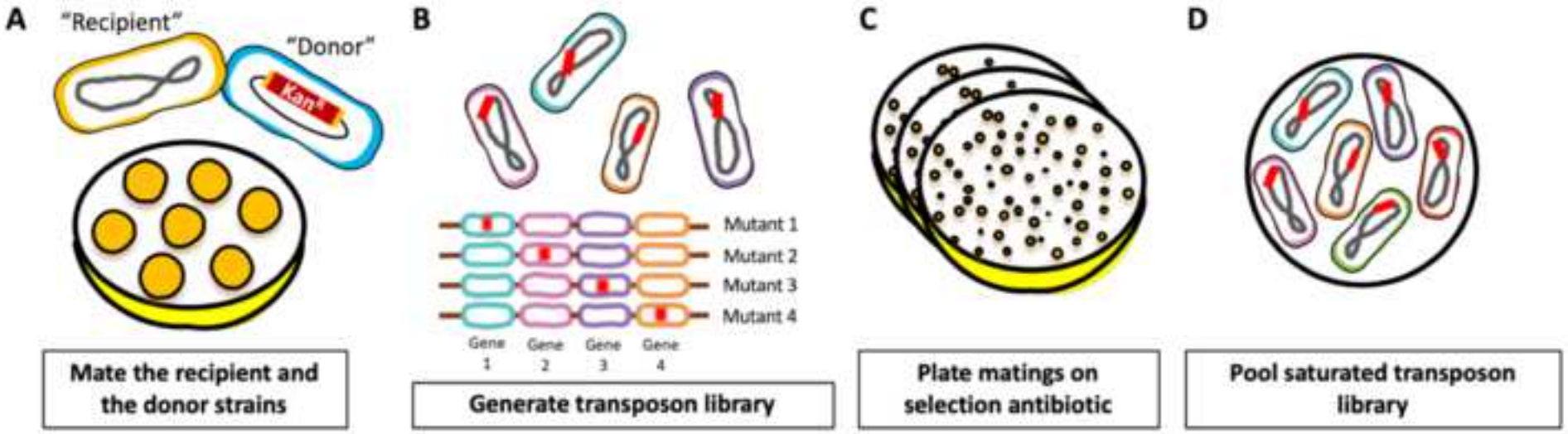

Figure 2

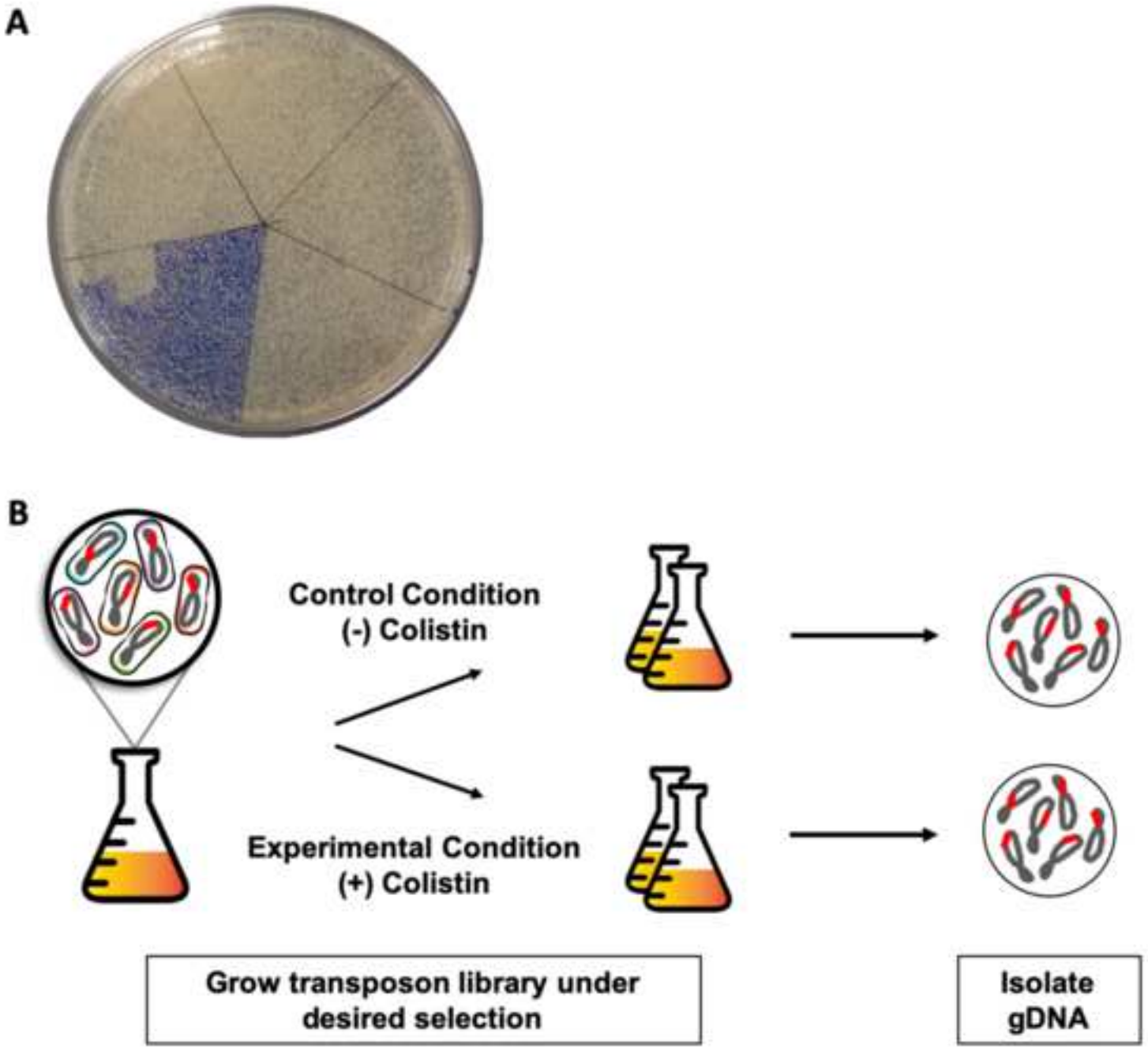

Figure 3

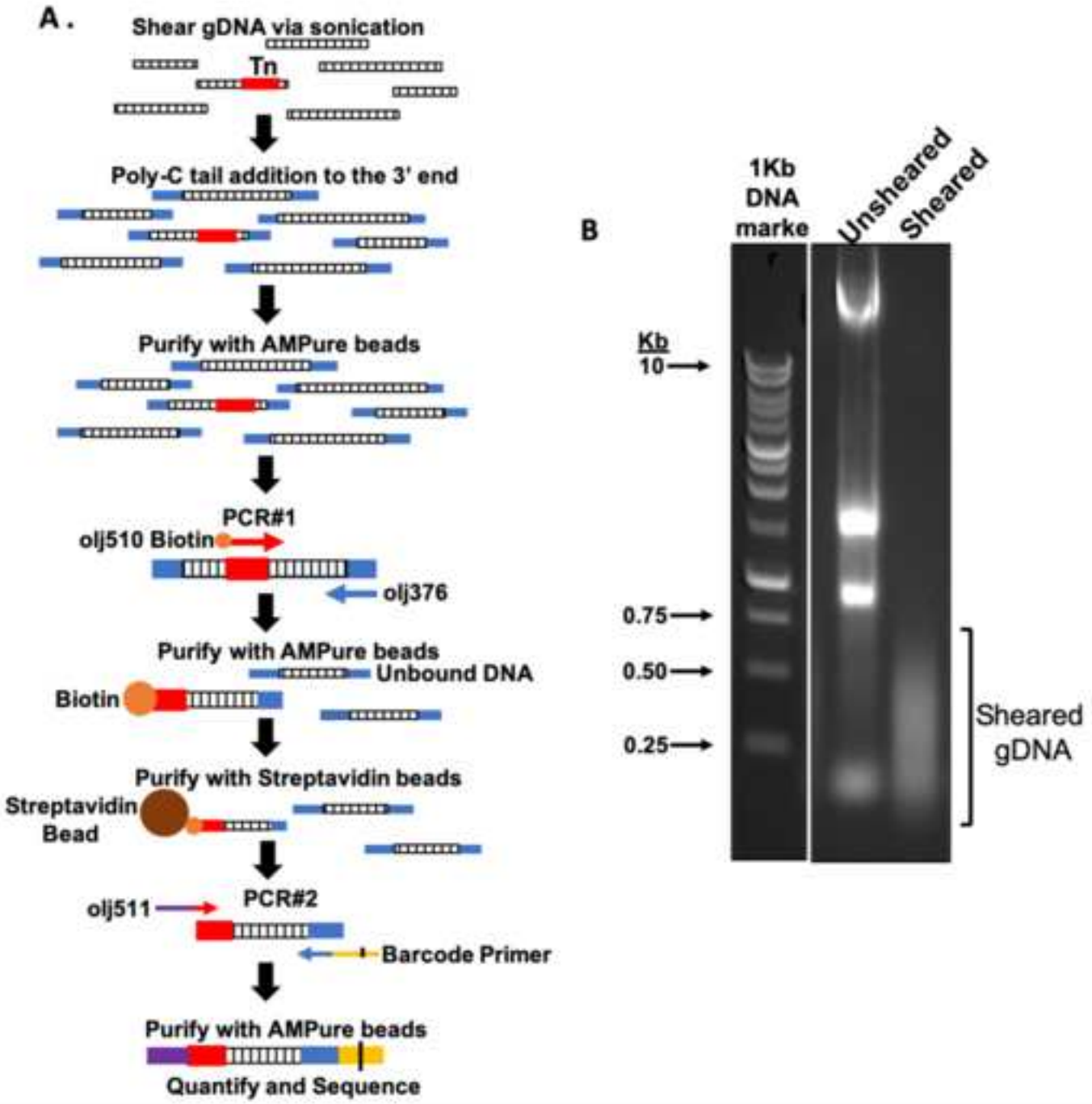

Figure 4

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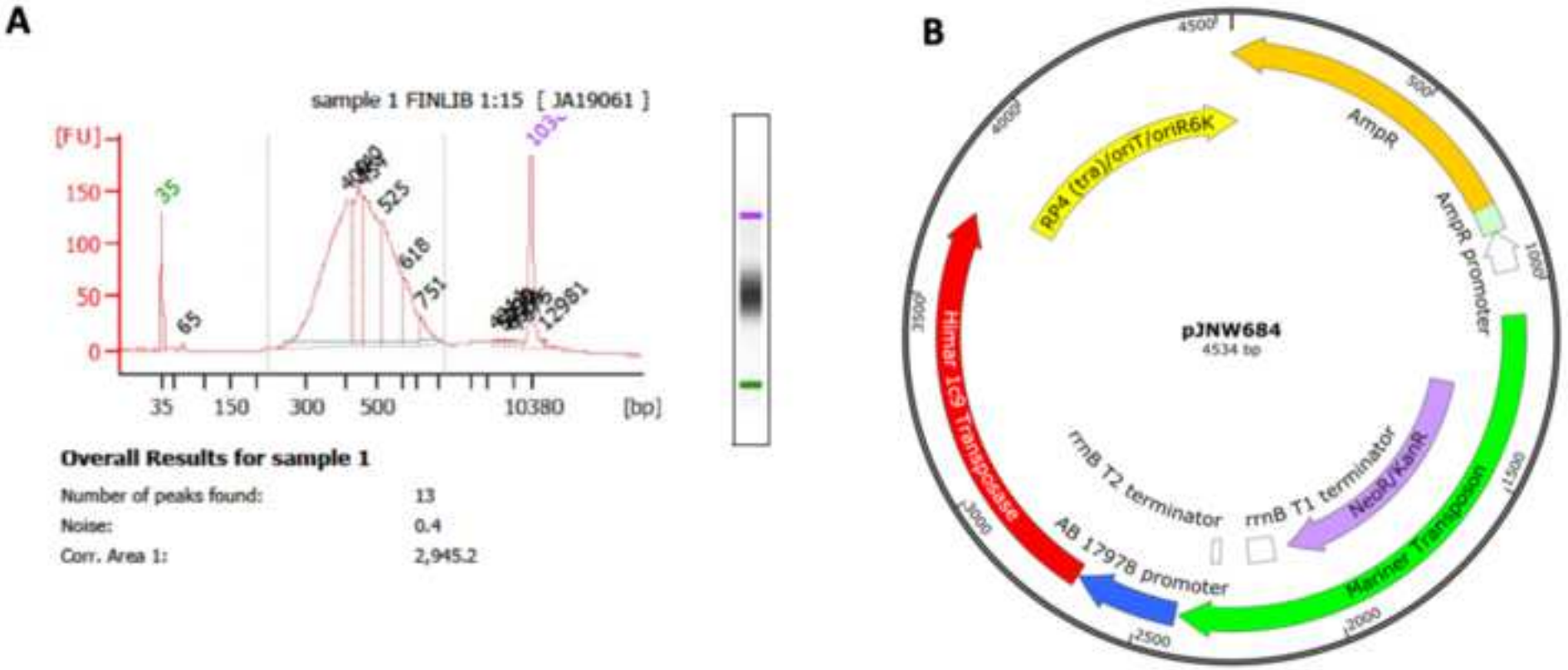

Table 1: Reaction setup. Setup conditions for poly-C, PCR 1 and PCR 2 reactions.

Reaction	**Setup**	Conditions
Poly-C reaction	30 µL of sheared gDNA 2.5 µL of 9.5 mM dCTP/0.5 mM ddCTP 10 µL of 5X Terminal deoxynucleotidyl transferase (Tdt) reaction buffer 1.25 µL of rTdt 6.25 µL of water to 50 µL	Incubate at 37ºC for 1 hour
PCR 1	23 µL 3'-poly-C purified DNA (entire sample from previous step) 10 µL 10x high-fidelity DNA polymerase reaction mix 2 µL 10 mM dNTPs 2 µL 50 mM MgSO4 1 µL 30 µM olj 510 biotin 3 µL 30 µM olj 376 0.5 µL high-fidelity DNA polymerase 8.5 µL pure water to 50 µL total	1 cycle: 2 min 94 ºC 15 cycles: 15 s 94 ºC 30 s 60 ºC 2 min 68 ºC 1 cycle: 4 min 68 ºC Hold: 4 ºC
PCR 2	DNA bound beads from previous step 10 µL 10x high-fidelity DNA polymerase reaction mix 2 µL 10 mM dNTP 2 µL 50 mM MgSO4 1 µL 30 µM olj 511 1 µL 30 µM barcode primer (Table 2) 0.5 µL high-fidelity DNA polymerase 33.5 µL pure water to 50 µL	1 cycle: 2 min 94 ºC 15 cycles: 15 s 94 ºC 30 s 60 ºC 2 min 68 ºC 1 cycle: 4 min 68 ºC Hold: 4 ºC

Table 2: PCR Amplification Primers. PCR amplification primers used in the protocol to amplify the transposon junctions. The purpose of each is listed in the first column.

Purpose	**Name**	**Sequence**
Anneals to transposon	olj510-Biotin	Biotin-GATGGCCTTTTTGCGTTTCTACCTGCAGGGCGCG
Anneals to poly-C tail	olj376	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGG
Nested to transposon + P5 adapter: P5 capture site – *P5 sequencing site*– N5 – **Tn**	olj511	AATGATACGGCGACCACCGA*GATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT* NNNNN**GGGGACTTATCATCCAACCTGTTAG**
Nested to olj376: P7 sequencing site – barcode **XXXXXX** – *P7 capture site*) ***see **Table 2** for specific barcodes***	BC##	*CAAGCAGAAGACGGCATACGAGAT* **xxxxxx**GTGACTGGAGTTCAGACGTGTG

Table 3: Barcode Primers. Barcode primers are used in the second PCR step to amplify the transposon

Primer	Read	Barcode	Sequence
BC1	ATCACG	**CGTGAT**	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTG
BC2	CGATGT	**ACATCG**	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTG
BC3	TTAGGC	**GCCTAA**	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTG
BC4	TGACCA	**TGGTCA**	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAGTTCAGACGTGTG
BC5	ACAGTG	**CACTGT**	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAGTTCAGACGTGTG
BC6	GCCAAT	**ATTGGC**	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAGTTCAGACGTGTG
BC7	CAGATC	**GATCTG**	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGACGTGTG
BC8	ACTTGA	**TCAAGT**	CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAGTTCAGACGTGTG
BC9	GATCAG	**CTGATC**	CAAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAGTTCAGACGTGTG
BC10	TAGCTT	**AAGCTA**	CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTG
BC11	GGCTAC	**GTAGCC**	CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTG
BC12	CTTGTA	**TACAAG**	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTG
BC13	AGTCAA	**TTGACT**	CAAGCAGAAGACGGCATACGAGAT**TTGACT**GTGACTGGAGTTCAGACGTGTG
BC14	AGTTCC	**GGAACT**	CAAGCAGAAGACGGCATACGAGAT**GGAACT**GTGACTGGAGTTCAGACGTGTG
BC15	ATGTCA	**TGACAT**	CAAGCAGAAGACGGCATACGAGAT**TGACAT**GTGACTGGAGTTCAGACGTGTG
BC16	CCGTCC	**GGACGG**	CAAGCAGAAGACGGCATACGAGAT**GGACGG**GTGACTGGAGTTCAGACGTGTG
BC17	GTAGAG	**CTCTAC**	CAAGCAGAAGACGGCATACGAGAT**CTCTAC**GTGACTGGAGTTCAGACGTGTG
BC18	GTCCGC	**GCGGAC**	CAAGCAGAAGACGGCATACGAGAT**GCGGAC**GTGACTGGAGTTCAGACGTGTG
BC19	GTGAAA	**TTTCAC**	CAAGCAGAAGACGGCATACGAGAT**TTTCAC**GTGACTGGAGTTCAGACGTGTG
BC20	GTGGCC	**GGCCAC**	CAAGCAGAAGACGGCATACGAGAT**GGCCAC**GTGACTGGAGTTCAGACGTGTG
BC21	GTTTCG	**CGAAAC**	CAAGCAGAAGACGGCATACGAGAT**CGAAAC**GTGACTGGAGTTCAGACGTGTG
BC22	CGTACG	**CGTACG**	CAAGCAGAAGACGGCATACGAGAT**CGTACG**GTGACTGGAGTTCAGACGTGTG
BC23	GAGTGG	**CCACTC**	CAAGCAGAAGACGGCATACGAGAT**CCACTC**GTGACTGGAGTTCAGACGTGTG
BC24	GGTAGC	**GCTACC**	CAAGCAGAAGACGGCATACGAGAT**GCTACC**GTGACTGGAGTTCAGACGTGTG
BC25	ACTGAT	**ATCAGT**	CAAGCAGAAGACGGCATACGAGAT**ATCAGT**GTGACTGGAGTTCAGACGTGTG
BC26	ATGAGC	**GCTCAT**	CAAGCAGAAGACGGCATACGAGAT**GCTCAT**GTGACTGGAGTTCAGACGTGTG
BC27	ATTCCT	**AGGAAT**	CAAGCAGAAGACGGCATACGAGAT**AGGAAT**GTGACTGGAGTTCAGACGTGTG
BC28	CAAAAG	**CTTTTG**	CAAGCAGAAGACGGCATACGAGAT**CTTTTG**GTGACTGGAGTTCAGACGTGTG
BC29	CAACTA	**TAGTTG**	CAAGCAGAAGACGGCATACGAGAT**TAGTTG**GTGACTGGAGTTCAGACGTGTG
BC30	CACCGG	**CCGGTG**	CAAGCAGAAGACGGCATACGAGAT**CCGGTG**GTGACTGGAGTTCAGACGTGTG
BC39	CTATAC	**GTATAG**	CAAGCAGAAGACGGCATACGAGAT**GTATAG**GTGACTGGAGTTCAGACGTGTG
BC40	CTCAGA	**TCTGAG**	CAAGCAGAAGACGGCATACGAGAT**TCTGAG**GTGACTGGAGTTCAGACGTGTG
BC42	TAATCG	**CGATTA**	CAAGCAGAAGACGGCATACGAGAT**CGATTA**GTGACTGGAGTTCAGACGTGTG
BC43	TACAGC	**GCTGTA**	CAAGCAGAAGACGGCATACGAGAT**GCTGTA**GTGACTGGAGTTCAGACGTGTG
BC44	TATAAT	**ATTATA**	CAAGCAGAAGACGGCATACGAGAT**ATTATA**GTGACTGGAGTTCAGACGTGTG
BC45	TCATTC	**GAATGA**	CAAGCAGAAGACGGCATACGAGAT**GAATGA**GTGACTGGAGTTCAGACGTGTG
BC46	TCCCGA	**TCGGGA**	CAAGCAGAAGACGGCATACGAGAT**TCGGGA**GTGACTGGAGTTCAGACGTGTG
BC47	TCGAAG	**CTTCGA**	CAAGCAGAAGACGGCATACGAGAT**CTTCGA**GTGACTGGAGTTCAGACGTGTG
BC48	TCGGCA	**TGCCGA**	CAAGCAGAAGACGGCATACGAGAT**TGCCGA**GTGACTGGAGTTCAGACGTGTG

Table of Materials

Click here to access/download;Table of Materials;Copy of D2_R1_JoVE_Materials_equipment (2).xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10 mM ddCTP, 2',3'-Dideoxycytidine-5'-Triphosphate	Affymetrix	77112	
100 mM dCTP 2'-Deoxycytidine-5'-Triphosphate	Invitrogen	10217-016	
100bp DNA Ladder Molecular Weight Marker	Promega	PR-G2101	
100mm x 15mm Petri Dishes	Corning	351029	
150mm x 15mm Petri Dishes	Corning	351058	
1X B&W	N/A	N/A	Dilute 2X B&W by half to get 1X B&W.
2,6-Diaminopimelic acid	Alfa Aesar	B2239103	used at 600 μM
2X B&W	N/A	N/A	Add 2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) in water. Used with Streptavidin beads. Solutions keep at room temperature.
50mL Conical Sterile Polypropylene Centrifuge Tubes	Fisher Scientific	12-565-271	
9.5 mM dCTP/0.5 mM ddCTP	N/A	N/A	9.5 μl 100 mM dCTP; 5 μl 10 mM ddCTP; 85.5 μl water. Store at -20°C.
AccuPrimeTM Pfx DNA Polymerase	Invitrogen	12344	
Acinetobacter baumannii ATCC 17978	ATCC	N/A	Amp^S, Kan^S
Ampicillin (100 mg/L)	Fisher Scientific	BP1760	used at 100 mg/L
AMPure XP PCR purification system	BECKMAN	A63881	
BioAnalyzer	Agilent	G2939B	
Bioanalyzer High Sensitivity DNA Analysis	Agilent	5067-4626	
Deoxynucleotide Solution Mix (dNTP)	New England	N0447L	
DynaMag-2 Magnetic rack	Invitrogen	12321D	
E.coli MFD Dap-	N/A	N/A	DAP Auxotroph, requires 600 μM exogenously added DAP to grow. Contains RP4 machinery for plasmid transfer. Carrier for JNW68 (36).
Ethanol	Fisher Scientific	A4094	
Externally Threaded Cryogenic Vials	Corning	09-761-71	
Glass beads	Corning	72684	
Glycerol	Fisher Scientific	G33	
Inoculating loops	Fisher Scientific	22-363-602	Scraping tool
Kanamycin	Fisher Scientific	BP906	used at 25 mg/L
LB agar, Miller	Fisher Scientific	BP1425	
LB broth, Miller	Fisher Scientific	BP1426	
LoTE	N/A	N/A	Add 3 mM Tris-HCl, 0.2 mM EDTA (pH 7.5) in water. Used with Streptavidin beads. Solutions keep at room temperature.
Lysis buffer	N/A	N/A	9.34 mL TE buffer; 600 μl of 10% SDS; 60 μl of proteinase K (20 mg/mL)
Phenol/Chloroform/Isoamyl Alcohol (25:24:1 Mixture, pH 6.7/8.(	Fisher Scientific	BP1752I	
Phosphate Buffered Saline, 10X Solution	Fisher Scientific	BP39920	Diluted to 1X
Qubit 4 Fluorometer	Thermo Fisher	Q33238	
Qubit Assay Tubes	Thermo Fisher	Q32856	
Qubit dsDNA HS Assay Kit	Thermo Fisher	Q32851	
Sonicator with refridgerated waterbath	Qsonica Sonicator:	Q2000FCE	
Streptavidin Magnetic Beads	New England	S1420S	
TE buffer	N/A	N/A	10 mM Tris-HCl (pH 8.0); 1 mM EDTA (pH 8.0)
Terminal Deoxynucleotidyl Transferase (rTdt)	Promega	PR-M1875	

Editorial comments:
All Author comments are highlighted in blue below. All referenced changes are also highlighted blue in the revised manuscript.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. We proofread the manuscript and have corrected all spelling/grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. We formatted the manuscript according to the changes suggested. Paragraph indentations were corrected to 0 for left and right and none for special, and line spacing was corrected to single throughout the manuscript.

3. Please make the title concise and remove redundancy (i.e. words like comprehensive approach). We updated the title to "A streamlined method to generate saturating transposon insertion libraries in Gram-negative bacteria for high-throughput sequencing" **(lines 2 - 3).**

4. Please define all abbreviations during the first-time use. We defined all abbreviations at the time of first use, including names of bacterial species.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol. We ensured that the long Abstract is within the required word limit (**212 words**) and states the goal of the protocol clearly within the required word limit **(lines 26 - 44).**

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Qubit, BioAnalyzer, etc. We removed all commercial language in the manuscript to more generic terms. Specifically, Qubit was changed to fluorometer, BioAnalyzer to chip-based capillary electrophoresis, AMPure beads to size-selection paramagnetic beads, Accuprime pfx DNA polymerase to high fidelity DNA polymerase and Qsonica ultrasonic processor Q2000 with refrigerated component to sonicator.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." We corrected all text to the imperative tense. Any text which could not be written in the imperative tense has been added as a "**NOTE**"

8. The Protocol should contain only action items that direct the reader to do something. We ensured that the Protocol only contains action item directing the reader to do something.

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step. We ensured that each step in the protocol section only contains 2-3 action items per step.

10. Please ensure you answer the "how" question, i.e., how is the step performed? We ensured the "how" question is answered for each step.

11. Please ensure that you use complete sentences throughout. We used complete sentences throughout.

12. Some of the details such as reaction set up, thermocycler conditions etc can be moved to a table in .xlsx format and uploaded separately to your editorial manager account. Please do not embed the table in the manuscript. We moved reaction setup and thermocycler condition details into a excel spreadsheet format (**Table 1**). No tables are embedded in the manuscript.

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. We verified the Protocol section is within the required 10-page limit. The authors highlighted 2.75 pages or less of the Protocol identifying the essential steps for the video portion of the publication.

14. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The results in the manuscript are described in the context of the described techniques, including steps on analyzing resulting data and specific findings.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." We ensured all the figures are original for this publication and are not being reused from a previous publication.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique We proofread the manuscript to ensure the

Discussion section meets the requirements, including critical steps, modifications, significance and potential applications of the method.

17. Please remove the figure legends from the uploaded figures. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. We removed all figure legends from the uploaded figure files and ensured that all the figure legends are included at the end of the Representative Results section in the manuscript.

18. Please sort the materials table in alphabetical order. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. We sorted the Table of Equipment and Materials in alphabetical order and removed any trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:

The authors describe a protocol for creating a saturating transposon mutant library in *A. baumannii*. The authors provide step by step instructions for how to culture the donor and recipient strains up through the development and purification of the sequencing library. The method described yields more mutants as compared to previously described protocols and the use of mechanical DNA shearing was done as opposed to enzymatic methods. The protocol would be a useful resource for the community.

Major Concerns:

1. By using the DNA shearing method, the authors have minimized several steps but besides this, they should mention the benefits of the quality. Faster is good, but the quality is also important. We thank the reviewer for their suggestion to mention benefits of quality gained by using the mechanical DNA shearing method described in this manuscript. We expanded the text to outline benefits of this approach, such as requiring smaller amounts of starting material while increasing consistency of the results, reducing risk of DNA cross contamination, limiting loss of sample and yielding high-quality reads that are longer (50 nucleotides) than reads obtained using other methods (14-16 nucleotides). Together, these improvements lead to more effective and precise mapping of sequences and more robust downstream analyses. The authors have included text in the Discussion section to address this concern (**lines 498 - 500, 503 - 513**).

2. The author should mention if the primers used for generating the library are designed for a particular sequencing instrument (Illumina, etc). We thank the reviewer for pointing out this missing piece of information. The authors have expanded the text to more clearly indicate the use of the Illumina sequencing platform. This was addressed in the Representative Results (**lines 397 - 99**) and the Discussion (**line 511 - 12**) sections of the manuscript.

3. The authors should provide guidance for how to analyze the sequencing data. The authors provide detailed steps up until the library is sequenced. However, the library is of limited value without the downstream analysis. We thank the reviewer for this important comment and critique. To address the reviewer's concern, the authors have included a paragraph in the Representative Results section detailing Illumina sequencing parameters and downstream analysis of sequencing data (**lines 402 – 417**).

4. Figure 3A is difficult to read. We thank the reviewer for their observation of Figure 3A. To address this concern, the authors have increased the font size and bolded all text in Figure 3A.

Minor Concerns:

1. Line 75-77 - The author should soften the language and not use "eliminated". It is true that polymyxins use decreased because of the availability of newer and better alternatives. However, the drug was not "eliminated". It was always present. We thank the reviewer for pointing out this inconsistency. The authors have softened the language by updating text in the Introduction to say that polymyxin use in the clinic was "limited" instead of "eliminated" (**line 76 - 77**).

2. Line 156 - The author should consider suggesting that the mating be done at 1:1, 1:10, or 10:1 donor:recipient ratios. This will be helpful for others who want to adapt the protocol beyond ATCC 17978Should cultures be measured by OD to confirm equivalent CFUs? We thank the reviewer for their suggestion to include donor : recipient ratios used for the mating. The authors have added that mating is done as a 10:1 donor : recipient ratio as a "**NOTE**" in the Protocol section (**line 165**).

3. Line 234- Why 13,333 colonies per plate? This seems oddly specific. Why not 1E4? We thank the reviewer for asking this important question. To address this concern, the authors added a "NOTE" in the Protocol section explaining that the CFU count was determined to be ~ 10^5 CFU/mL, and based on that the mating volume was adjusted to get 13,333 colonies per plate as this would provide ample number of colonies on 30 plates for a high-resolution mutant library (~ 400,000 mutants) without overcrowding the plates (**lines 212 - 14**).

4. Line 286-296 - It would be helpful to explain why the cultures are seeded at OD600 = 0.001. Why not add a fixed volume to each culture and subculture to OD600 = 0.50? We thank the reviewer for their suggestion to explain why the cultures are seeded at OD_{600} = 0.001 during the challenge growth experiment. To take this into consideration, the authors have included a "NOTE" in the Protocol section explaining that it is important to catch the bacteria in log phase, and since different strains may have different generation times under various growth conditions, normalizing the starting inoculum by seeding all cultures at a fixed OD_{600} helps monitor and regulate the replication of each culture to ensure that the cultures replicate as many times as possible while still harvesting in log phase **(lines 256 – 263)**. Line 286- It would be best practice to dilute the bacteria in 1 mL of PBS and not LB. The bacteria

are on ice, but the use of 4. PBS will further prevent bacterial growth. We thank the reviewer for this comment and have altered the text to indicate PBS, not LB **(line 249).**

5. Line 340 - Does there need to be mixing of the 70% EtOH with DNA pellet via pipette? We thank the reviewer for this question. To clarify, the authors have modified text in the Protocol section to direct the reader to "wash" the DNA pellet with 70 % ethanol "by pipetting" (**lines 292 – 93**).

Reviewer #2:

Overview - This manuscript demonstrates how mariner transposon mutagenesis can be used to analyze the whole genome of Acinetobacter baumannii to detect genes that provide a selective advantage for colistin tolerance.

Introduction

After Line 105 - Introduce the transposon here, since it is the main tool for this technique. We thank the reviewer for their suggestion to describe the transposon in the Introduction. To address the reviewer's comment, the authors introduced the plasmid, PJNW684 in the Introduction section and detailed use of the *Himar1 mariner* transposon system and construction of the plasmid pJNW684 (**lines 106 – 127**).

Protocol

Line 122 - Comment: Streaking each strain out on agar plates and then using single colonies that grow may also eliminate the potential for contamination. We thank the reviewer for this important comment. To correct, the authors updated the Protocol section directing the reader to streak out each strain on agar plates and using single isolated colonies to inoculate each subsequent liquid culture (**lines 144 – 150**).

Line 164 - About how many plates are required? 5 drops per plate = 500 ul per plate. If there about 5 ml of mating mixture, then there should be 10 plates? We thank the reviewer for their question. The authors addressed this by including a "**NOTE**" in the Protocol section that outlines the number of plates required if there are 5 droplets/plate and if there are 7 droplets/plate (**lines 169**).

Line 174 - If there are 10 plates and bacteria from each plate is resuspended with 1.5 ml LB, then the final volume is about (1.5 X 10) + 5 = 20 ml? We thank the reviewer for their question. The authors have included a "**NOTE**" in the Protocol section clarifying that the total final volume is 20 mL for this step (**line 176**).

Line 185 - It says "pellet matings." If all the plates were combined, it should be singular, "mating." We thank the author for pointing out the inconsistency. The text was corrected to the singular form, "mating" (**lines 178, 180**).

Line 236 - Should "beads" be "rod?" Will this require 400,000/13,333 = 30 plates? We thank the reviewer for their question and for pointing out the miscalculation in the number of plates

required for this step. The authors have corrected the number of plates required for this step of the protocol to "30" (**line 214**) and added a "**NOTE**" in the Protocol section to address the use of beads versus rods (**lines 215 – 216**).

Line 240 - You can refreeze the mating? A. baumannii can withstand freezing and thawing but E. coli and many other bacteria cannot. This should be noted for novices because several of my grad students have had problems reviving their frozen stock cultures. It is always because they did not know that thawing and refreezing kills most frozen stock cultures. We thank the reviewer for their important comment and critique. We have modified the Protocol section instructing the reader to initially freeze the mating in 1 mL aliquots (**line 190**), then to thaw just one aliquot of the frozen mating for generating the final bacterial mutant library (**line 208**), and then to "dispose" of the used tube containing excess mating (**line 219**). Additionally, we added a "**NOTE**" in the Protocol section explaining that freeze/thaw cycles can add selective pressure on bacterial cultures, which can skew downstream results, so it is best practice to use a fresh aliquot each time (**lines 220 – 21**).

Lines 254 and 259 - 30 plates X 5 ml LB = 150 ml. This will require at least three-50 ml tubes? We thank the reviewer for the question. We corrected the text to reflect use of multiple 50 mL conical tubes for this step of the protocol and clarified this in a "**NOTE**" as well (**line 234**).

Lines 270 - 279 - Why are there 4 flasks, instead of just 2 flasks? Is this just because the experiment is being done in duplicate? To make things simpler, why not put 100 ml in a two 500 ml flasks. We thank the reviewer for their questions. To clarify why there are 4 flasks, instead of just 2, we have included a "**NOTE**" in the Protocol section explaining that the challenge growth experiment described in the manuscript is performed in biological duplicates so the experimental setup requires 4 x 250 mL Erlenmeyer flasks, 2 x 250 mL flasks per condition tested (**line 245 – 47**).

Representative Results

Lines 523-531 - This paragraph would be better in the Introduction because the manuscript is based on the transposon in plasmid, pNJW684. Be sure to emphasize that only the transposon is inserted into the host chromosome and not the whole plasmid. A novice might not immediately realize this. Also, insertion of the transposon alone and not the whole plasmid containing the transposase genes makes the insertion stable so that the transposon does not move to new locations after insertion. We thank the reviewer for their suggestion. To address this, we have expanded the Introduction to include a paragraph describing the mariner-family transposon system used in this manuscript encoded on pJNW684 and detailing the mechanism of transposon mutagenesis using pJNW684 and emphasizing that only the transposon and not the entire plasmid is inserted in the recipient chromosome to provide single, stable insertions (**lines 106 – 127**).

Line 537 - Approximately, how many CFUs per ml? We thank the reviewer for their question. The authors have indicated that approximately 10^5 CFUs/mL were calculated from the collective mating **(lines 212, 383).**

Line 552 - This is probably due to page limitations, but this section leaves the reader hanging. What were the results after sequencing? How many genes were detected in the untreated sample? How many genes were detected in the treated sample? How many genes appear to be required for colistin-tolerance and how was this determined? What was the identity of some of these genes? Before trying this technique, a reader needs to be reassured that it can be successful. We thank the reviewer for this important comment and critique. To address this, we have expanded the Representative Results section to include details about results after sequencing, which yielded 30 million reads/ sample providing 62.5-fold coverage of the transposon library. We have also provided insight for the downstream analysis of sequencing data, which, for example, identified the PmrAB two-component system to be essential for *A. baumannii* survival at sub-inhibitory concentrations of colistin, as well as other genes known to be important for the resistance phenotype (**lines 402 – 417**).

Lines 594-597 - Table 2 seems like an afterthought. The purpose of bar codes are not clearly explained. What is the purpose of a P7 sequencing site? In the case of this experiments, would you use two or four barcodes? We thank the reviewer for pointing out this missing information. We have addressed this in the Representative Results section by explaining that the P7 sequencing site is required for binding to the Illumina flow cell and the six-base barcode is used for demultiplexing libraries post-sequencing (**lines 396 - 99**). Additionally, we included text in the Protocol section to indicate that one barcode per sample was used for the experiment described in this manuscript (**lines 365 – 66**).

Discussion

Lines 602-604 - Maybe put this sentence at the end of the Discussion and expand on it. At this point, readers want to know if this technique can be used in their research. Explain what modifications need to be made for using it with other strains of bacteria and describe examples. We thank the reviewer for their suggestion to demonstrate the versatility and applicability of this method. To address this comment, we have added text in the Introduction to describe plasmid modifications that allow for it to be used in other bacterial species (**lines 120 – 125**). We have also expanded the Discussion section to include several examples found in literature where Tn-seq was used to discover important information about diverse bacterial species and conditions (**lines 470 – 479**).

Lines 607-612 - This paragraph is redundant by just summarizing the whole procedure and can be deleted. Instead, a paragraph that extends on the first paragraph should be added. Explain whether the techniques identified genes for proteins already known to participate in colistin tolerance. What new genes were identified? How is better than other methods such as microarrays, 2-D gel electrophoresis, qPCR etc.? We thank the reviewer for their valuable critique and suggestions. We have addressed this by deleting the redundant paragraph and

supplementing it with text outlining the benefits of this approach over other methods (**lines 480 – 488**).

Line 615 - If another reader would like to use this technique on a different bacterial strain and a different resistance phenotype, what modification, if any, would need to made to pNJW684? We thank the reviewer for their recommendation to explain the broad applicability of this method. We have addressed this by including text in the Introduction describing the modifications that can be made to the transposon system used in this manuscript for use in various other bacterial species and strains (**lines 121 – 127**).

Lines 623-626 - Does shearing the DNA using sonication cause overhangs? Since poly C tails are added, does it matter if overhangs are caused by shearing? We thank the reviewer for their important question. We addressed this comment by explaining that DNA shearing via sonication may generate overhangs but the overhangs should not alter the library build because it relies on exogenously added poly-C tail to prime DNA synthesis (**lines 508 – 512**).

Figure 3

A) Is the green circle below "PCR #1" biotin? The orange circle is clearly labeled as biotin. What is the difference between the two circles? We thank the reviewer for pointing out this inconsistency in Figure 3A. We corrected the color of the circle below “PCR#1” to orange to match the orange circle that is labeled as biotin. We also bolded the text and increased the font size.

B) Purified genomic DNA is usually larger than 10 kb. Why is the unsheared DNA only about 2 kb in size? We thank the reviewer for observing our oversight in Figure 3B. We updated this figure to include the entire gel image showing purified genomic DNA which is larger than 10 Kb.

Figure 4

1) Because the transposon is the main tool for this procedure, maybe it should be the first figure in the manuscript and explained in the introduction (see above). We agree with the reviewer that the transposon should be explained in the introduction and addressed this concern **(lines 106-120).** However, we did not move the transposon map to Figure 1 because we do not reference specific genes in pJNW684 until the Representative results section. We think manuscript flows better in this order. If the reviewer insists on moving, we can alter.

2) Please remove the white block over "Himar _____ transposase." We thank the reviewer for pointing this out we have removed the white block “Himar1c9 transposase.”

3) Please explain how this plasmid is transferred when it does not contain any tra or mob genes. pJNW does encode RP-4 *tra* components. The feature was added to the plasmid map in Figure 4 - labeled as RP4 (*tra*)/oriT/oriR6K.

4) What genes are present in the E. coli MFD Dap- strain that allows for transfer? MFD*pir* contains an integrated RP4-based transfer machinery, which allows plasmid transfer – this detail was added to table of strain descriptions.